

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/82, 15/29, C07K 14/415, A01N 63/02, A01H 5/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 95/19443</b> <b>(43) International Publication Date:</b> 20 July 1995 (20.07.95)
<b>(21) International Application Number:</b> PCT/IB95/00002 <b>(22) International Filing Date:</b> 3 January 1995 (03.01.95) <b>(30) Priority Data:</b> 08/181,271 13 January 1994 (13.01.94) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 08/181,271 (CIP) Filed on 13 January 1994 (13.01.94) <b>(71) Applicant (for all designated States except US):</b> CIBA-GEIGY AG [CH/CH]; Klybeckstrasse 141, CH-4002 Basle (CH). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> RYALS, John, A. [US/US]; 14 Sanderling Court, Durham, NC 27713 (US). ALEXANDER, Danny, C. [US/US]; 217 Midenhall Way, Cary, NC 27513 (US). UKNES, Scott, J. [US/US]; 1003 Pinedale Drive, Apex, NC 27502 (US). WARD, Eric, R. [US/CH]; Säckingerstrasse 20, CH-4058 Basle (CH). <b>(74) Common Representative:</b> CIBA-GEIGY AG; Patentabteilung, Klybeckstrasse 141, CH-4002 Basle (CH).		<b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES AND USES THEREOF		
<b>(57) Abstract</b> <p>The present invention provides recombinant or chimaeric DNA molecules comprising plant SAR genes, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis independent or in a protein-synthesis dependent manner. Methods for obtaining these DNA molecules are also provided. Additionally, the present invention provides chemically inducible wheat genes, Arabidopsis chitinase IV, Maize PR-1mz, and Maize thaumatin PR-5mz. The anti-pathogenic sequences according to the invention can be genetically engineered and transformed into plants to obtain transgenic plants with enhanced resistance to disease. In one embodiment of the present invention, the chemically regulatable DNA promoter sequence of the Arabidopsis Pr-1 gene is provided. Another aspect of the present invention is a method of improving protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins exert a synergistic effect.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

**CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES**  
**AND USES THEREOF**

The present invention relates to DNA molecules encoding proteins capable of conferring plant disease and/or plant pest resistance. Another aspect of the invention relates to the chemical regulation of gene expression. In particular, this invention relates to non-coding DNA sequences which, in the presence of chemical regulators, regulate the transcription of associated DNA sequences in plants. Both aspects of the invention relate, in part, to genes associated with the response of plants to pathogens.

Advances in recombinant DNA technology coupled with advances in plant transformation and regeneration technology have made it possible to introduce new genetic material into plant cells, plants or plant tissue, thus introducing new traits, e.g., phenotypes, that enhance the value of the plant or plant tissue. Recent demonstrations of genetically engineered plants resistant to pathogens (EP-A 240 332 and EP-A 223 452) or insects (Vaeck, M. *et al.*, *Nature* 328: 33 (1987)) and the production of herbicide tolerant plants (DeBlock, M. *et al.*, *EMBO J.* 6: 2513 (1987)) highlight the potential for crop improvement. The target crops can range from trees and shrubs to ornamental flowers and field crops. Indeed, it is clear that the "crop" can also be a culture of plant tissue grown in a bioreactor as a source for some natural product.

Various methods are known in the art to accomplish the genetic transformation of plants and plant tissues (i.e., the stable introduction of foreign DNA into plants). These include transformation by Agrobacterium species and transformation by direct gene transfer.

A. tumefaciens is the etiologic agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms, that results in the formation of tumors or galls in plant tissue at the site of infection. Agrobacterium, which normally infects the plant at wound sites, carries a large extrachromosomal element called the Ti (tumor-inducing) plasmid.

Ti plasmids contain two regions required for tumorigenicity. One region is the T-DNA (transferred-DNA) which is the DNA sequence that is ultimately found stably transferred to plant genomic DNA. The other region required for tumorigenicity is the vir (virulence) region which has been implicated in the transfer mechanism. Although the vir region is absolutely required for stable transformation, the vir DNA is not actually transferred to the infected plant. Transformation of plant cells mediated by infection with Agrobacterium tumefaciens

- 2 -

and subsequent transfer of the T-DNA alone have been well documented. See, for example, Bevan, M.W. and Chilton, M-D., *Int. Rev. Genet.* 16: 357 (1982).

After several years of intense research in many laboratories, the Agrobacterium system has been developed to permit routine transformation of a variety of plant tissue. Representative species transformed in this manner include tobacco, tomato, sunflower, cotton, rapeseed, potato, soybean, and poplar. While the host range for Ti plasmid transformation using A. tumefaciens as the infecting agent is known to be very large, tobacco has been a host of choice in laboratory experiments because of its ease of manipulation.

Agrobacterium rhizogenes has also been used as a vector for plant transformation. This bacterium, which incites hairy root formation in many dicotyledonous plant species, carries a large extrachromosomal element called an Ri (root-inducing) plasmid which functions in a manner analogous to the Ti plasmid of A. tumefaciens. Transformation using A. rhizogenes has developed analogously to that of A. tumefaciens and has been successfully utilized to transform, for example, alfalfa, Solanum nigrum L., and poplar.

Several so-called direct gene transfer procedures have been developed to transform plants and plant tissues without the use of an Agrobacterium intermediate (see, for example, Koziel *et al.*, *Biotechnology* 11: 194-200 (1993) herein incorporated by reference). In the direct transformation of protoplasts the uptake of exogenous genetic material into a protoplast may be enhanced by use of a chemical agent or electric field. The exogenous material may then be integrated into the nuclear genome. The early work was conducted in the dicot tobacco where it was shown that the foreign DNA was incorporated and transmitted to progeny plants, see e.g. Paszkowski, J. *et al.*, *EMBO J.* 3: 2717 (1984); and Potrykus, I. *et al.*, *Mol. Gen. Genet.* 199: 169 (1985).

Monocot protoplasts have also been transformed by this procedure in, for example, Triticum monococcum, Lolium multiflorum (Italian ryegrass), maize, and Black Mexican sweet corn.

Alternatively exogenous DNA can be introduced into cells or protoplasts by microinjection. A solution of plasmid DNA is injected directly into the cell with a finely pulled glass needle. In this manner alfalfa protoplasts have been transformed by a variety of plasmids, see e.g. Reich, T.J. *et al.*, *Bio/Technology* 4: 1001 (1986).

- 3 -

A more recently developed procedure for direct gene transfer involves bombardment of cells by microprojectiles carrying DNA, see Klein, T.M. *et al.*, *Nature* 327: 70 (1987). In this procedure tungsten particles coated with the exogenous DNA are accelerated toward the target cells, resulting in at least transient expression in the example reported (onion).

Just as there is a variety of methods for the transformation of plant tissue, there is a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated. In recent years it has become possible to regenerate many species of plants from callus tissue derived from plant explants. The plants which can be regenerated from callus include monocots, such as corn, rice, barley, wheat and rye, and dicots, such as sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from tissue transformed with A. tumefaciens has been demonstrated for several species of plants. These include sunflower, tomato, white clover, rapeseed, cotton, tobacco, and poplar. The regeneration of alfalfa from tissue transformed with A. rhizogenes has also been demonstrated. Plant regeneration from protoplasts is a particularly useful technique, see Evans, D.A. *et al.*, in: "Handbook of Plant Cell Culture", Vol. 1, MacMillan Publ. Co., 1983, p. 124. When a plant species can be regenerated from protoplasts, then direct gene transfer procedures can be utilized, and transformation is not dependent on the use of A. tumefaciens. Regeneration of plants from protoplasts has been demonstrated for rice, tobacco, rapeseed, potato, eggplant, cucumber, poplar, and corn.

Various plant tissues may be utilized for transformation with foreign DNA. For instance, cotyledon shoot cultures of tomato have been utilized for Agrobacterium mediated transformation and regeneration of plants (see European application EP-A 249432). Further examples include Brassica species (see WO 87/07299) and woody plant species, particularly poplar (see U.S. Patent No. 4,795,855, incorporated by reference herein in its entirety).

The technological advances in plant transformation and regeneration technology highlight the potential for crop improvement via genetic engineering. There have been reports of genetically engineered tobacco and tomato plants which are resistant to infections of, for example, tobacco mosaic virus (TMV) and resistant to different classes of herbicides. Insect resistance has been engineered in tobacco and tomato plants.

- 4 -

The potential for genetic engineering is not limited to field crops but includes improvements in ornamentals, forage crops and trees. A less obvious goal for plant biotechnology, which includes both genetic engineering and tissue culture applications, is the enhanced production of a vast array of plant-derived chemical compounds including flavors, fragrances, pigments, natural sweeteners, industrial feedstocks, antimicrobials and pharmaceuticals. In most instances these compounds belong to a rather broad metabolic group, collectively denoted as secondary products. Plants may produce such secondary products to ward off potential predators, attract pollinators, or combat infectious diseases.

Plant cell cultures can be established from an impressive array of plant species and may be propagated in a bioreactor. Typical plant species include most of those that produce secondary products of commercial interest. It has been clearly demonstrated in a number of agriculturally important crop plants that stable genetic variants arising from the tissue culture of plant somatic cells (somaclonal variation) can be induced and selected. Numerous advantages flow from plant tissue culture production of secondary compounds. These include (1) the possibility of increased purity of the resultant product, (2) the conversion of inexpensive precursors into expensive end products by biotransformation, and (3) the potential for feeding substrate analogs to the culture to create novel compounds.

Whether the target of genetic engineering of plants is a field crop, ornamental shrub, flower, tree or a tissue culture for use in a bioreactor, a principal advantage to be realized is the control of expression of the chimeric gene so that it is expressed only at the appropriate time and to the appropriate extent, and in some situations in particular parts of the plant. For example, in order to achieve a desirable phenotype the chimeric gene may need to be expressed at levels of 1% of the total protein or higher. This may well be the case for fungal resistance due to chimeric chitinase expression or insect resistance due to increased proteinase inhibitor expression. In these cases the energy expended to produce high levels of the foreign protein may result in a detriment to the plant whereas, if the gene were expressed only when desired, for instance when a fungal or insect infestation is imminent, the drain on energy, and therefore yield, could be reduced.

Alternatively, the phenotype expressed by the chimeric gene could result in adverse effects to the plant if expressed at inappropriate times during development. For example, if the chimeric gene product were a plant hormone that induced pod abscission, early expression could bring about abscission of the fruit from the plant before the seed had

- 5 -

matured, resulting in decreased yield. In this case it would be much more advantageous to induce the expression of this type of gene to a time when pod abscission is preferred, or least injurious to the plant.

For tissue in culture or in a bioreactor the untimely production of a secondary product could lead to a decrease in the growth rate of the culture resulting in a decrease in the yield of the product. Therefore, it would be advantageous to allow the culture to grow without expressing the secondary product and then induce the chimeric gene at an appropriate time to allow for an optimized expression of the desired product.

In view of considerations like these, as well as others, it is clear that control of the time, extent and/or site of expression of the chimeric gene in plants or plant tissues would be highly desirable. Control that could be exercised easily in a field, a greenhouse or a bioreactor would be of particular commercial value.

External manipulation of the expression of endogenous genes which contain chemically regulatable sequences by the application of a chemical regulator is already known from Ward, E. *et al.*, *Plant Cell* 3: 1085-1094 (1991); Williams *et al.*, *Bio/Technology* 10: 540-543 (1992); and Uknes, S. *et al.*, *Plant Cell* 5: 159-169 (1993). The control provided is somewhat limited, however, due to the responsiveness of such sequences to endogenous chemical metabolites and cell signals as well as externally applied chemical regulators. In one aspect of the invention, alterations are taught which block the responsiveness of these genes to endogenous signals while maintaining responsiveness to externally applied chemical regulators.

Pest infestation of crop plants causes considerable loss of yield throughout the world and most crops grown in the U.S. suffer infestation, particularly from insects. Major insect pests in the U.S. include the European Corn Borer (*Ostrinia nubilalis*) in maize, the cotton bollworm (*Heliothis zea*) and the pink bollworm (*Pectinophora gossypiella*) in cotton and the tobacco budworm (*Heliothis virescens*) in tobacco. Resistance to pests is difficult to achieve using conventional breeding programs and typically pests have been controlled using chemical pesticides.

Recent advances in molecular biology and plant transformation technology have demonstrated the possibility of expressing in transgenic plants genes encoding insecticidal proteins; this represents a novel approach in the production of crop plants resistant to

- 6 -

pests. Most notably, the expression of genes encoding the *Bacillus thuringiensis* endotoxin has been successful in a wide range of plant species, and the analysis of transgenic lines expressing such genes has been well documented (Vaech *et al.*, *Nature* 328: 33-37 (1987); Fischhoff *et al.*, *Biotechnology* 5: 807-813 (1987); Carozzi *et al.*, *Plant Mol. Biol.* 20: 539-548 (1992); Koziel *et al.*, *Biotechnology* 11: 194-200 (1993)). Other insecticidal genes have been used successfully in generating insect resistant transgenic plants.

One approach has been the overexpression of genes encoding insect enzyme inhibitors such as trypsin inhibitors or seed proteins with known insecticidal activity, such as lectins (Hilder *et al.*, *Nature* 330: 160-163 (1987)). Indeed, plants expressing both the cowpea trypsin inhibitor and pea lectin were shown to have additive effects in providing insect resistance (Boulter *et al.*, *Crop Protection* 9: 351-354 (1990)). In cases where pests are able infest parts of the plant or tissues not readily accessible to conventional pesticides, a transgenic approach may be more successful than the use of conventional pesticides.

For example, the tobacco budworm *Heliothis* is well known to be difficult to control using pesticides because it burrows deep into the plant tissue. Additionally, some pests of roots, such as nematodes, are not readily controlled by foliar applications of pesticides. An advantage in the use of transgenic plants expressing insecticidal proteins is the controlled expression of the proteins in all desired tissues.

Chitinases catalyze the hydrolysis of chitin, a  $\beta$ -1,4-linked homopolymer of *N*-acetyl-D-glucosamine. Several different plant chitinases have been described and the cDNA sequences for some of these have been reported (Meins *et al.*, *Mol. Gen. Genet.* 232: 460-469 (1992)). Based on structural characteristics, three classes have been distinguished. Class I chitinases have two structural domains, a cysteine-rich amino-terminal hevein domain and a carboxyterminal catalytic domain separated from the former by a variable spacer. Class II chitinases lack the cysteine-rich hevein domain and all or part of the variable spacer, but retain the catalytic domain. Class III chitinases lack the hevein domain and contain a catalytic domain that shares no significant homology with that of the class I or class II enzymes.

Class I chitinase gene expression is induced by ethylene, whereas class II and class III chitinase gene expression is induced in the SAR response. The chitinase/lysozyme disclosed in European patent application EP-A 392 225 and the chitinase/lysozyme disclosed in SEQ ID Nos. 20 and 21 are class III chitinases. It is well known that the level of chitinase activity of plants increases dramatically after pathogen invasion (Mauch *et al.*,



- 7 -

*Plant Physiol.* 76: 607-611 (1984)) and this is presumably due to the host plant's attempts to degrade the chitin of the fungal cell wall. Furthermore, chitinase has been shown *in vitro* to inhibit fungal and insect growth, and in transgenic plants a bacterial chitinase has been shown to exhibit inhibitory effects towards numerous pathogens and pests including insects (Suslow & Jones WPO 90-231246; U.S. Pat. Nos. 4,940,840 and 4,751,081; herein incorporated by reference in their entirety).

For over 90 years, scientists and naturalists have observed that when plants survive pathogen infection they develop an increased resistance to subsequent infections. In 1933, a phenomenon termed "physiological acquired immunity" was described in an extensive literature review by Chester, K.S., *Quart. Rev. Biol.* 8: 275-324 (1933). At that time, scientists believed they were investigating a phenomenon analogous to the immune response in mammals. In retrospect, at least three different processes were being called "acquired immunity": viral cross protection, antagonism (or biocontrol), and what we now refer to as systemic acquired resistance (SAR).

The first systematic study of SAR was published by A. Frank Ross in 1961. Using tobacco mosaic virus (TMV) on local lesion hosts, Ross demonstrated that infections of TMV were restricted by a prior infection. This resistance was effective against not only TMV, but also tobacco necrosis virus and certain bacterial pathogens. Ross coined the term "systemic acquired resistance" to refer to the inducible systemic resistance (Ross, A.F., *Virology* 14: 340-358 (1961)) and "localized acquired resistance" (LAR) to describe the resistance induced in inoculated leaves (Ross, A.F., *Virology* 14: 329-339 (1961)). It is still unclear whether SAR and LAR are aspects of the same response or distinct processes.

In the past 30 years, SAR has been demonstrated in many plant species and the spectrum of resistance has been broadened to include not only viruses and bacteria, but also many agronomically important phytopathogenic fungi (see Kuc, J., *BioScience* 32: 854-860 (1982)). However, understanding of the biochemical events leading to the establishment of SAR had not progressed substantially until the past dozen years. In 1982, the accumulation of a group of extracellular proteins called pathogenesis-related (PR) proteins were reported to correlate with the onset of SAR (Van Loon, L.C. *et al.*, *Neth. J. Plant. Path.* 88: 237-256 (1982)). In 1979, salicylic acid (SA) and certain benzoic acid derivatives were reported to be able to induce both resistance and the accumulation of PR proteins (White,

- 8 -

R.F., *Virology* 99: 410-412 (1979). As a result, SA was considered as a possible endogenous signal molecule (Van Loon, L.C. *et al.*, *Neth. J. Plant. Path.* 88: 237-256 (1982)). However, progress slowed through the 1980's and the involvement of PR proteins and salicylic acid in SAR was questioned.

With the advent of genetic engineering and recombinant DNA technology, the possibility of manipulating genetic material to improve the phenotype of plants has arisen. The present invention is based in part upon the discovery of the identity and role of genes involved in SAR which has allowed the application of modern molecular biological techniques for improved plant disease and plant pest resistance.

There are two major aspects of the present invention. The first aspect relates to chemically regulatable DNA sequences and the chemicals which regulate them. The second aspect relates to plant pathogenesis-related proteins. Both aspects of the invention have arisen, in part, from the inventors' identification and characterization of cDNAs and corresponding genes involved in the plant response to pathogen infection.

A principal object of the present invention is to provide additional means for chemically regulating the expression of a desired gene in a plant, seed, or plant tissue. In particular, the present invention relates to identification and isolation of a non-coding DNA sequence obtainable from the promoter region of an *Arabidopsis* PR-1a gene which, in the presence of chemical regulators, regulate the transcription of associated DNA sequences in plants.

Another principal object of the present invention is to provide transgenic plants, transgenic seeds and the progeny thereof expressing levels of plant pathogenesis-related anti-pathogenic proteins such as tobacco chitinase/lysozyme according to the invention, or substantially homologous proteins, which confer an enhanced disease-resistant and/or pest-resistant phenotype with respect to wild-type plants.

Accordingly, to meet this objective and others, the second aspect of the present invention disclosed herein provides for the isolation, cloning and identification of novel cDNA clones coding for plant pathogenesis-related (PR) proteins. These cDNAs, or their genomic counterparts, or DNA molecules with substantial homology to either (all of the above collectively referred to herein as "anti-pathogenic sequences"), can be engineered for expression of the encoded PR proteins or anti-sense RNA and transformed into plants to confer enhanced resistance or tolerance to various diseases and/or pests as described

herein. These DNA molecules may be engineered for constitutive expression, expression in particular tissues or at certain developmental stages, or induced expression in response to an inducer, for example in response to a chemical inducer as described herein.

The present invention further provides novel methods for differential screening and enriching for induced cDNAs, particularly those cDNAs induced in response to pathogen infection or a chemical inducer which triggers a response mimicking pathogen infection.

The present invention teaches the cloning of SAR genes by differential screening of tissues induced and non-induced to the systemic acquired response. SAR induction was found cause the transcription of genes in a protein synthesis-dependent fashion and also a protein synthesis-independent fashion. Two methods were used to clone specifically genes whose induced transcription is protein-synthesis independent:

Firstly, recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis independent manner, are obtained by

- (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
- (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
- (c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

Thus, cDNAs which were cloned by standard differential screening techniques were further screened on SAR-induced RNA isolated with and without cycloheximide (CHX) pre-treatment.

Secondly, recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis independent manner, are obtained by

- (a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;

- 10 -

- (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
- (c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

Thus, a PCR-based "differential display" technique is used to identify SAR-induced, but protein synthesis independent cDNAs directly.

Differential display RNAs were prepared with and without SAR induction and CHX treatment. CHX as an inhibitor of protein synthesis is well known in the art and described by Greenberg *et al.*, *Mol. Cell Biol.* 6: 1050-1057 (1986), Lau and Nathans, *Proc. Natl. Acad. Sci.* 84: 1182-1186 (1987), and Uknes *et al.*, *Plant Cell* 5: 159-169 (1993). The methods described provide recombinant or chimaeric DNA molecules comprising plant SAR genes and preferably plant SAR cDNA molecules, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis independent manner, that is in the presence of a protein synthesis inhibitor such as cycloheximide. Thus, a number of genes were cloned which were induced by the SAR response, yet expressed independently of protein synthesis. These cloned genes are likely signal transducers in the pathway leading from induction to the development of the resistant state.

Additionally recombinant or chimaeric DNA molecules are provided which comprise plant SAR genes and preferably plant SAR cDNA molecules, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis dependent manner, that is only in the absence of a protein synthesis inhibitor such as cycloheximide. Recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis dependent manner, can be obtained by

- (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
- (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor such as cycloheximide; and

- 11 -

- (c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

Alternatively, these molecules can be obtained by

- (a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
- (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor such as cycloheximide; and
- (c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

#### **DESCRIPTION OF GENE SEQUENCES**

- SEQ ID No. 1: The cDNA sequence encoding an *Arabidopsis* class IV chitinase with a hevein domain (pChit4-TA).
- SEQ ID No. 2: The cDNA sequence encoding an *Arabidopsis* class IV chitinase without a hevein domain (pChit4-TB).
- SEQ ID No. 3: The cDNA sequence of the wheat gene WCI-1.
- SEQ ID No. 4: The partial cDNA sequence of the 5' end of the wheat gene WCI-2 which encodes a lipoxygenase isozyme. The partial sequence of the 3' end of this cDNA is provided in SEQ ID No. 5.
- SEQ ID No. 5: The partial cDNA sequence of the 3' end of the wheat gene WCI-2 which encodes a lipoxygenase isozyme. The partial sequence of the 5' end of this cDNA is provided in SEQ ID No. 4.
- SEQ ID No. 6: The cDNA sequence of the wheat gene WCI-3.
- SEQ ID No. 7: The cDNA sequence of the wheat gene WCI-4.
- SEQ ID No. 8: The cDNA sequence of the wheat gene WCI-5.
- SEQ ID No. 9: A tobacco protein-synthesis dependent gene involved in the regulation of the systemic acquired resistance response designated p1.1.1.

- 12 -

- SEQ ID No. 10: A tobacco protein-synthesis dependent gene involved in the regulation of the systemic acquired resistance response designated p11.31.4.
- SEQ ID No. 11: The 5' DNA sequence of a tobacco protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated p11.30.13.
- SEQ ID No. 12: The DNA sequence of the 3'end of the same protein-synthesis independent gene described in Seq. ID. No. 11 cloned from tobacco and involved in the regulation of the systemic acquired resistance response designated p11.30.13. This sequence is derived from the non-coding strand (*i.e.* the "bottom" strand). The first base listed is therefore located in the furthest 3' position.
- SEQ ID No. 13: A tobacco protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated p1.4.3. This sequence is identical to the thioredoxin gene published by Brugidou *et al.*, *Mol. Gen. Genet.* 238: 285-293 (1993).
- SEQ ID No. 14: A protein-synthesis independent SAR gene cloned from tobacco designated p66B1.
- SEQ ID No. 15: A protein-synthesis dependent SAR gene cloned from tobacco designated p14.22.3.
- SEQ ID No. 16: An *Arabidopsis* protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated pDPA2.
- SEQ ID No. 17: The cDNA sequence encoding an Arabidopsis PR-1 protein cloned into plasmid pAPR1C-1.
- SEQ ID No. 18: Oligo B used in example 4A
- SEQ ID No. 19: Oligo C used in example 4A
- SEQ ID No. 20: The cDNA sequence encoding a basic tobacco chitinase/lysozyme protein cloned into the plasmid pBSCL2.
- SEQ ID No. 21: The cDNA sequence encoding an acidic tobacco chitinase/lysozyme protein cloned into the plasmid pBSTCL226.
- SEQ ID No. 22: A representative molecular adaptor sequence.
- SEQ ID No. 23: Oligonucleotide primer for the PR-1 gene.
- SEQ ID No. 24: Oligonucleotide primer for GUS gene.

- 13 -

- SEQ ID No. 25: Oligonucleotide primer for the AHAS gene.
- SEQ ID No. 26: Oligonucleotide primer for the BT gene.
- SEQ ID No. 27: Peptide sequence used to design a degenerate forward oligonucleotide sequence for PCR cloning of chitinase IV
- SEQ ID No. 28: Peptide sequence used to design a degenerate reverse oligonucleotide sequence for PCR cloning of chitinase IV
- SEQ ID No. 29: The cDNA sequence of the Maize PR1-like gene PR-1mz
- SEQ ID No. 30: Amino acid sequence of the protein encoded by SEQ ID No. 29
- SEQ ID No. 31: The cDNA sequence of the Maize thaumatin-like gene PR-5mz
- SEQ ID No. 32: Amino acid sequence of the protein encoded by SEQ ID No. 31
- SEQ ID No. 33: The cDNA sequence of the Arabidopsis gene PSI-1
- SEQ ID No. 34: The cDNA sequence of the Arabidopsis gene PSI-2
- SEQ ID No. 35: The cDNA sequence of the Arabidopsis gene PSI-3
- SEQ ID No. 36: The cDNA sequence of the Arabidopsis gene PSI-4
- SEQ ID No. 37: The cDNA sequence of the Arabidopsis gene PSI-5

### **DEFINITIONS**

In order to provide a clear and consistent understanding of the specification and the claims, including the scope given to such terms, the following definitions are provided:

**Anti-pathogenic Sequence:** A DNA molecule encoding a plant pathogenesis-related (PR) protein, or a DNA molecule with substantial homology thereto, which is capable of conferring enhanced resistance or tolerance to disease and/or pests when expressed in a plant, seed, or plant tissue.

**Anti-sense Mechanism:** A mechanism for regulation of gene expression based on the presence in a cell of a RNA molecule complementary to at least a portion of the mRNA encoded by the gene. This mechanism is thought to involve controlling the rate of translation of mRNA to protein due to the presence in a cell of an RNA molecule complementary to at least a portion of the mRNA being translated.

Associated DNA Sequence: A DNA sequence whose cellular activity either (1) regulates the activity of another DNA sequence or (2) is regulated by another DNA sequence. This definition specifically embraces, but is not limited to, sequences which are physically adjacent in a continuous DNA strand or which are physically separated. Physical separation includes, for example, separation within the same DNA strand, location within different DNA strands, or discontinuous interspersed sequences (e.g., alternating regulatable and coding sequences) in one strand.

Chemically Regulatable DNA Sequence: A DNA sequence which is capable of regulating the transcription of an associated DNA sequence where the regulation is dependent on a chemical regulator. The sequences may be of natural or synthetic origin.

Chemically Regulatable Gene: A gene containing at least one non-coding chemically regulatable DNA sequence and at least one associated coding DNA sequence. The genes may be of natural, synthetic or partially natural/partially synthetic origin.

Chemical Regulator (for a chemically regulatable DNA sequence): An elemental or molecular species which controls (e.g., initiates, terminates, increases or reduces), by direct or indirect action, the activity of a chemically regulatable DNA sequence in a system in which the chemical regulator is not normally found in an active form in an amount sufficient to effect regulation of transcription, to the degree and at the time desired, of a transcribable DNA sequence associated with the chemically regulatable DNA sequence. This terminology is intended to embrace situations in which no or very little regulator is present at the time transcription is desired or in which some regulator is present but increased or decreased regulation is required to effect more or less transcription as desired.

Thus, if the system containing the chemically regulatable DNA sequence is a plant, for example a transgenic plant, a chemical regulator is a species not naturally found in the plant in an amount sufficient to effect chemical regulation, and thus transcription of an associated gene, to the desired degree at the time desired.

By "direct action" it is intended that the chemical regulator action result from the direct interaction between the chemical regulator and the DNA sequence. By "indirect action" it is meant that the regulator action results from the direct interaction between the chemical regulator and some other endogenous or exogenous component in the system,



- 15 -

the ultimate result of that direct interaction being activation or suppression of the activity of the DNA sequence. By "active form" it is intended that the chemical regulator be in a form required to effect control.

Chimeric Sequence or Gene: A DNA sequence containing at least two heterologous parts, e.g., parts derived from naturally occurring DNA sequences which are not associated in their naturally occurring states, or containing at least one part that is of synthetic origin and not found in nature.

Coding DNA Sequence: A DNA sequence which, when transcribed and translated, results in the formation of a cellular polypeptide.

Constitutive transcription: Transcription of substantially fixed amounts of a DNA sequence, irrespective of environmental conditions.

Gene: A discrete chromosomal region which is responsible for a discrete cellular product.

Inducers: Molecules that cause the production of larger amounts of macromolecules, compared to the amounts found in the absence of the inducer.

Inducible Protein: Proteins whose rate of production can be increased by the presence of inducers in the environment.

Non-coding DNA Sequence: A DNA sequence, which is not transcribed and translated, resulting in the formation of a cellular polypeptide when associated with a particular coding DNA sequence. A sequence that is non-coding when associated with one coding sequence may actually be coding when associated with another coding or non-coding sequence.

Phenotypic Trait: An observable property resulting from expression of a gene.

Plant Tissue: Any tissue of a plant in planta or in culture. This term includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures and any groups of plant cells organized into structural and/or functional units. The

use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

PR, or Pathogenesis-Related Proteins: Proteins expressed in plants reacting hypersensitively towards pathogens. This term embraces, but is not limited to, SAR8.2a and SAR8.2b proteins, the acidic and basic forms of tobacco PR-1a, PR-1b, PR-1c, PR-1', PR-2, PR-2', PR-2'', PR-N, PR-O, PR-O', PR-4, PR-P, PR-Q, PR-S, and PR-R major proteins, cucumber peroxidases, basic cucumber peroxidase, the chitinase which is a basic counterpart of PR-P or PR-Q, and the beta-1,3-glucanase (glucan endo-1,3-β-glucosidase, EC 3.2.1.39) which is a basic counterpart of PR-2, PR-N or PR-O, the pathogen-inducible chitinase from cucumber, class IV chitinases with and without a hevein domain, and the WCI ("Wheat Chemically Induced") gene proteins from . A hypersensitive reaction is characterized by a local necrosis of the tissues immediately surrounding the infection site of the pathogen and a subsequent localization of the pathogen, which is in contrast to a sensitive reaction wherein the pathogen spreads throughout the plant. Pathogens are, for example, viruses or viroids, e.g. tobacco or cucumber mosaic virus, ringspot virus or necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato bushy stunt virus, and like viruses, fungi, e.g. *Phytophthora parasitica* or *Peronospora tabacina*, bacteria, e.g. *Pseudomonas syringae* or *Pseudomonas tabaci*, or aphids, e.g. *Myzus persicae*. This list is not limiting in any respect.

Regulation: The increasing (inducing) or decreasing (repressing) of the level of expression of a gene or the level of transcription of a DNA sequence. The definition is not intended to embrace any particular mechanism.

Substantially Pure DNA Sequence: A DNA molecule (sequence) isolated in substantially pure form from a natural or non-natural source. Such a molecule may occur in a natural system, for example, in bacteria, viruses or in plant or animal cells, or may be provided, for example, by synthetic means or as a cDNA. Substantially pure DNA sequences are typically isolated in the context of a cloning vector. Substantially pure means that DNA sequences other than the ones intended are present only in marginal amounts, for example less than 5%, less than 1%, or preferably less than 0.1%. Substantially pure DNA sequences and

- 17 -

vectors containing may be, and typically are, provided in solution, for example in aqueous solution containing buffers or in the usual culture media.

Substantial Sequence Homology: Substantial sequence homology means close structural relationship between sequences of nucleotides or amino acids. For example, substantially homologous DNA sequences may be 80% homologous, preferably 90% or 95% homologous, and substantially homologous amino acid sequences may typically be 50% homologous, or more. Homology also includes a relationship wherein one or several subsequences of nucleotides or amino acids are missing, or subsequences with additional nucleotides or amino acids are interdispersed.

### ABBREVIATIONS

The following abbreviations are used herein:

AHAS	acetoxyhydroxyacid synthase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pair
BT	<u>Bacillus thuringiensis</u> endotoxin
BTH	methyl benzo-1,2,3-thiadiazole-7-carboxylate
CAT	chloramphenicol acetyltransferase
CETAB	hexadecyltrimethylammonium bromide
CHX	cycloheximide
2,4-D	2,4-dichlorophenoxyacetic acid
DTT	dithiothreitol
dicamba	3,6-dichloro-2-methoxybenzoic acid
EDTA	ethylenediamine N,N,N',N'-tetraacetic acid
GUS	beta-l,3-glucuronidase
INA	isonicotinic acid
kb	kilo base pair
LUX	luciferase

- 18 -

MES	2-(N-morpholino)ethanesulfonic acid
MU	4-methyl umbelliferyl glucuronide
NOS	nopaline synthase
NPT	neomycin phosphotransferase
NRRL	designation for deposits made with the Agricultural Research Culture Collection, International Depositing Authority, 1815 N. University Street, Peoria, Illinois 61604
OCS	octopine synthase
PEG	polyethylene glycol
picloram	4-amino-3,5,6-trichloropicolinic acid
PR protein	Pathogenesis-related protein
SA	salicylic acid
SAR	Systemic Acquired Resistance
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TMV	tobacco mosaic virus
Tris-HCl	tris(hydroxymethyl)methylamine hydrochloride
WCI	Wheat Chemically Induced (gene nomenclature designation)

### **ANTI-PATHOGENIC SEQUENCES**

The present invention also embraces anti-pathogenic DNA sequences which are capable of conferring enhanced disease resistance or disease tolerance when expressed in a plant or plant tissue. This includes coding sequences for plant pathogenesis-related (PR) proteins as described herein and sequences with substantial homology to these coding sequences.

Included within the scope of the present invention, in addition to the sequences exemplified specifically below and enumerated in the sequence listing, are cDNA sequences which are equivalent to the enumerated sequences which encode the given plant pathogenesis-related protein, and cDNA sequences which hybridize with the enumerated sequences and encode a polypeptide having some degree of disease-resistant activity of the given plant pathogenesis-related protein (i.e. an anti-pathogenic sequence).

- 19 -

Equivalent cDNA sequences are those which encode the same protein even though they contain at least one different nucleotide from the enumerated sequence. As is well known in the art, the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins, different nucleotide sequences can code for a particular amino acid. Thus, the genetic code can be depicted as follows:

Amino Acid	Codon	Amino Acid	Codon
Phenylalanine (Phe)	TTK	Histidine (His)	CAK
Leucine (Leu)	XTY	Glutamine (Gln)	CAJ
Isoleucine (Ile)	ATM	Asparagine (Asn)	AAK
Methionine (Met)	ATG	Lysine (Lys)	AAJ
Valine (Val)	GTL	Aspartic acid (Asp)	GAK
Serine (Ser)	QRS	Glutamic acid (Glu)	GAJ
Proline (Pro)	CCL	Cysteine (Cys)	TGK
Threonine (Thy)	ACL	Tryptophan (Trp)	TGG
Alanine (Ala)	GCL	Arginine (Arg)	WGZ
Tyrosine (Tyr)	TAK	Glycine (Gly)	GGL
Termination signal	TAJ		

Key: Each 3-letter deoxynucleotide triplet codon corresponds to a trinucleotide of mRNA, having a 5'-end on the left and a 3'-end on the right. All DNA sequences given herein are those of the strand whose sequence corresponds to the mRNA sequence, with thymine substituted for uracil. The letters stand for the purine or pyrimidine bases forming the deoxynucleotide sequence as follows:

A = adenine; G = guanine; C = cytosine; T = thymine

X = T or C if Y is A or G

X = C if Y is C or T

Y = A, G, C or T if X is C

Y = A or G if X is T

W = C or A if Z is A or G

- 20 -

W = C if Z is C or T

Z = A, G, C or T if W is C

Z = A or G if W is A

QR = TC if S is A, G, C or T;

QR = AG if S is T or C

J = A or G

K = T or C

L = A, T, C or G

M = A, C or T

The above shows that the amino acid sequence of the instant plant pathogenesis-related proteins can be prepared using different nucleotide sequences encoding the same amino acid sequence of the proteins. Accordingly, the scope of the present invention includes such "equivalent nucleotide sequences."

cDNA sequences that hybridize with a given enumerated sequence and encode a polypeptide or protein having at least some degree of activity of the corresponding plant pathogenesis-related protein are those which exhibit substantial sequence homology, as defined hereinabove, with the enumerated Sequence such that it hybridizes with the latter under low stringency conditions. Such conditions are described in Examples 17 and 20 of European patent application EP-A-0 392 225. Proteins translated from these hybridizable cDNA sequences have different primary structures from proteins translated from the enumerated Sequences. However, their respective secondary structures are the same. Thus, the former proteins retain some degree of the biological activity (plant disease resistance) of the latter proteins and are considered as plant pathogenesis-related proteins also, e.g. "a PR-O protein," "a PR-N protein," etc. As used elsewhere herein, the term "activity" is referred to as the property of enhanced disease resistance or tolerance conferred upon a transgenic plant by virtue of its ability to express the DNA encoding the PR protein

It is contemplated by the invention that increased disease resistance or tolerance or increased scope of protection against a broader range of pathogens can be achieved by providing for the expression of more than one anti-pathogenic DNA sequence in a transgenic plant or plant tissue. The increased disease resistance thus achieved is due to a synergistic effect created by expressing a combination of anti-pathogenic sequences in the

- 21 -

same plant or plant tissue. Thus, a method is provided which improves protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins exert a synergistic effect. Preferably the DNA molecules encode anti-pathogenic proteins according to the invention. Due to synergy, the anti-pathogenic effect of a particular sequence may be more readily detected when combined with one or more other anti-pathogenic sequences than when present alone.

The present invention further includes chimeric genes and vectors comprising one or more anti-pathogenic sequences analogous to the chimeric genes and vectors comprising chemically regulatable sequences described in parts F and G above. Any desired promoter may be used in association with the anti-pathogenic sequences of the invention, including promoters which confer constitutive expression, promoters which confer expression in a particular tissue or subset of tissues and/or at a particular developmental stage, and promoters which confer chemically regulatable expression containing the noncoding sequences taught herein.

Also included as part of the present invention are plant tissues, plants and seeds comprising anti-pathogenic sequences analogous to those plant tissues, plants and seeds comprising chemically regulatable sequences described in part H above.

### **DIFFERENTIAL CLONING AND SCREENING TECHNOLOGY**

A method has been conceived and developed which will allow efficient enrichment of sequences present in one population of molecules in greater amounts than in another population. The method's greatest utility is in situations where the populations are very similar and the differentially present sequences represent a very small proportion of the population.

If two populations of clones are similar and one wishes to isolate those clones which are present in one population in higher amounts (i.e. "induced" or differentially regulated"), past techniques involved screening with probes from the two populations (+/- screening; St. John and Davis, *Cell* 16:443-452 (1979)), or enrichment of probes or mRNAs by hybridization and hydroxy-apatite (HAP) chromatography (Davis, *et al.*, *Proc. Natl. Acad. Sci, USA* 81: 2194-2198 (1984)). The first method has a demonstrated sensitivity limitation

in that only clones present in greater than about one in 2,000 will be detected. The second is laborious, technically difficult, and achieves enrichments of 20-50 fold at best.

The present method involves exploiting two recent developments in molecular technology: the polymerase chain reaction (Saiki *et al.*, *Science* 239:487-491 (1988)) and biotin-avidin chromatography [Stahl, *et al.*, *Nuc. Acids. Res.* 16: 3026-3038 (1988)]. The polymerase chain reaction (PCR) allows simple synthesis of large amounts of DNA of specified sequence. Biotin-avidin chromatography allows the efficient separation of molecules bearing a biotin affinity tag from those molecules which do not bear the tag.

In its general form, the technique consists of isolating single strands of cDNA representing two different populations ("induced" vs "uninduced"), but of opposite cDNA polarity for the two populations, i.e. one of "sense" polarity relative to mRNA's, and the other its complement, or "anti-sense", polarity relative to mRNA's. The isolated strands from the "induced" population would have no affinity tag, while the strands of opposite polarity from the "uninduced" populations would have stable affinity tags. When these two populations are hybridized together, hybrids will form between complementary strands present in the two populations. Those strands from the "induced" population which have no counterparts, or many fewer counterparts, in the "uninduced" population, remain single stranded.

Due to the presence of the affinity tag (in essence a handle) on the strands of the "uninduced" population molecules, those strands and, most importantly, any hybrid molecules can be removed from the mixture by affinity chromatography. This leaves only those "induced" molecules which are not significantly represented in the "uninduced" population. These "induced" molecules can then be cloned by standard means and serve as an enriched population from which to isolate "induced" clones; alternatively, the enriched molecules can be amplified individually and sequenced directly.

An alternate scheme is the same as described above except that it involves incorporating a labile affinity tag only on the "induced" population molecules, while the affinity tag on the "uninduced" population is stable. "Labile" in this case means that the affinity tag can be removed at will, or be altered at will in such a way that it no longer serves as an affinity tag. In this scheme all the molecules in the hybridization mixture could bind to the affinity matrix, but only those "induced" molecules that are not hybridized to a complementary "uninduced" counterpart could be selectively recovered from the matrix for subsequent cloning.



- 23 -

The advantage of the methods of the invention described above over those previously described is the ability to isolate those genes which are turned on only to low levels, in specific circumstances, and which may play a causative role in some important biological phenomenon.

The present invention teaches the cloning of SAR genes by differential screening of tissues induced and non-induced to the systemic acquired response SAR induction causes the transcription of genes in a protein synthesis-dependent fashion and also a protein synthesis-independent fashion. Two methods were used to clone specifically genes whose induced transcription is protein-synthesis independent. Firstly, cDNAs which were cloned by standard differential screening techniques were further screened on SAR-induced RNA isolated with and without cycloheximide (CHX) pre-treatment. Secondly, a PCR-based "differential display" technique was used to identify SAR-induced, but protein synthesis independent cDNAs directly. Differential display RNAs were prepared with and without SAR induction and CHX treatment. The use of CHX as an inhibitor of protein synthesis is well known in the art and is described by Greenberg *et al.*, *Mol. Cell. Biol.* 6: 1050-1057 (1986), Lau and Nathans, *Proc. Natl. Acad. Sci.* 84: 1182-1186 (1987), and Uknes *et al.*, *Plant Cell* 5: 159-169 (1993). Thus, a number of genes were cloned which were induced by the SAR response, yet expressed independently of protein synthesis. These cloned genes are likely signal transducers in the pathway leading from induction to the development of the resistant state.

**EXAMPLES****Example (i):           Ligation in Agarose**

Following restriction digestion of plasmid DNA and electrophoretic separation of the fragments on a low melting TAE gel, the bands containing appropriate fragments are precisely excised and heated to 65°C to melt the agarose 2-5 µl are added to 15 µl water and the solution is left at 65°C for 10 minutes. This solution is cooled to 37°C and left for five minutes to equilibrate to temperature 2 µl of 10 X ligase buffer (200 mM Tris, pH 8.0, 100 mM MgCl<sub>2</sub>, 100 mM DTT, 10 mM ATP) are added along with 1 µl T4 DNA ligase (New England BioLabs), and this solution is allowed to solidify and incubate at 15°C overnight.

**Example (ii):           Transformation From Agarose**

The agarose containing the appropriate DNA is melted by incubating at 65°C for 10 minutes 10 µl of this solution are added to 30 µl of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA), mixed and allowed to stand at room temperature. Frozen competent cells (*E. coli* strain DH5) are placed on wet ice to thaw. The diluted DNA solution is added to 200 µl of cells and allowed to stand on ice for 20 minutes. The cells containing the DNA are then heat-shocked for 90 seconds at 41°C. The cells are then left at room temperature for 10 minutes 0.8 ml of SOC medium (Hanahan, D., *J. Mol. Biol.* 166: 557-580 (1983)) is added and the culture is incubated at 37°C for one hour 100 µl of the culture is plated on LB plates (Miller, *supra*) containing 100 µg/ml ampicillin (L-amp) and the plates are incubated overnight at 37°C. Positive colonies are picked and restreaked to a second L-amp plate and the plates are incubated overnight at 37°C.

**Example (iii):           Labelling DNA Restriction Fragments**

DNA is treated with the appropriate restriction enzymes and fragments are separated by electrophoresis on a low-gelling temperature agarose gel. A band containing the fragment of interest is excised and the DNA purified by standard techniques 50 ng of the DNA fragment is labelled using the IBI Random primer kit "Prime time" according to the manufacturers directions.

- 25 -

**Example (iv): Southern Blotting**

3 µg of tobacco DNA is digested with various restriction enzymes under the conditions suggested by the supplier. The DNA is extracted with phenol, precipitated with ethanol and then resuspended in gel loading buffer (15% ficoll, 0.025% bromophenol blue, 10 mM EDTA, pH 8). Samples are loaded and electrophoresed on a 0.5% agarose gel at 5 V/cm until the bromophenol blue dye reaches the end of the gel. The DNA is transferred to Gene-Screen Plus (DuPont) using the alkaline transfer procedure as described by the supplier. Pre-hybridization, hybridization and washing are according to the manufacturer's recommendation. Hybridization is detected by autoradiography.

**Example (v): Molecular Adaptors**

A typical molecular adaptor for the conversion of a PstI site to a BamHI site is the sequence

5'-GGGATCCCTGCA-3' (SEQ ID No. 22).

This molecule is synthesized on an Applied Biosystems Synthesizer using B-cyano-ethylphosphoramidite chemistry and purified by reverse-phase HPLC. About 2 µg of this oligonucleotide is kinased according to Maniatis *et al.*, supra, p. 125. The oligonucleotide solution is heated to 65°C in a water bath and allowed to cool to room temperature over a period of about 30 minutes. An approximately 10-fold molar excess of this annealed adapter is added to the digested DNA along with 10 X ligase buffer, T4 DNA ligase, and an appropriate amount of water. A typical reaction is:

DNA to be adapted:	1-2 µl (~ 1 pmol)
Adapter:	1 µl (~10 pmol)
10 X ligase buffer:	1 µl
T4 DNA ligase:	1 µl
Water:	5-6 µl

This solution is incubated at 12-15°C for 30 minutes, and heated to 65°C for 30 minutes to inactivate the ligase. The salt concentration and volume are adjusted for the appropriate restriction digest and the adapted DNA is digested to expose the adapted

"sticky end." Unincorporated adapters are removed either by electrophoresis on an agarose gel or by sequential isopropanol precipitations.

**Example (vi):            *Primer Extension Mapping***

**A. Synthesis and 5' End Labeling of Primers for Primer Extension**

The following primer oligomers are synthesized using an Applied Biosystems Synthesizer and  $\beta$ -cyanoethylphosphoramidite chemistry:

PR-1:    5'-ATAGTCTTGTTGAGAGTT-3' (SEQ ID No. 23)

GUS:    5'-TCACGGGTTGGGGTTTCTAC-3' (SEQ ID No. 24)

AHAS:    5'-AGGAGATGGTTTGGTGGA-3' (SEQ ID No. 25)

BT:    5'-ATACGTTCTACTATCATAGT-3' (SEQ ID No. 26)

The oligonucleotides are purified by reverse-phase high pressure liquid chromatography (HPLC) 5 pmol of each oligo is kinased (Maniatis, T. *et al.*, supra, at p. 125) using 200  $\mu$ C of  $^{32}$ P-ATP (6000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ l). After incubation at 37°C for 30 minutes, the reaction is diluted to 100  $\mu$ l, extracted with phenol/chloroform and then precipitated three times with 50  $\mu$ g carrier RNA. The final precipitate is resuspended in 1 X reverse-transcriptase buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 3 mM MgCl<sub>2</sub>) at a concentration of 2 nM. The specific activity of the labeled oligonucleotide is determined to be about 3 X 10<sup>6</sup> Cvpmp/pmol.

**B. Total RNA Preparation**

Total RNA is prepared essentially as described by Lagrimini, L.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 84: 7542 (1987). Tissue is ground under liquid nitrogen in a mortar and pestle. The ground tissue is added to grinding buffer (Lagrimini *et al.*, supra) using 2.5 ml per gram tissue. An equal volume of phenol is then added and the emulsion is homogenized in a Brinkman polytron. A one-half volume of chloroform is added and the emulsion is gently mixed for 15 minutes. The phases are separated by centrifugation and

- 27 -

the aqueous phase is removed. RNA is precipitated by the addition of sodium acetate to 0.3 M and 2.5 volumes ethanol. The precipitate is collected by centrifugation and resuspended in 2 ml sterile water. Lithium chloride is added to a final concentration of 3 M and left at 4°C overnight. The precipitate is collected by centrifugation and the pellet is washed with ice-cold 80% ethanol. The pellet is dried and resuspended in 500 µl sterile water. The concentration of this total RNA preparation is determined spectrophotometrically.

Alternatively, RNA is extracted from callus as described above except that the callus tissue is cut into cubes approximately 3 mm in size, and added to pre-chilled mortars and pestles for grinding in liquid nitrogen prior to the polytron step.

### C. Primer Extension

50 µg of total RNA is lyophilized in a 500 µl Eppendorf tube. The RNA is resuspended in 30 µl of radiolabeled probe solution and heated to 70°C for 10 minutes. The tube is slowly cooled to 37°C and allowed to incubate overnight. Without removing the tube from the 37°C water bath, 2 µl of 10 X reverse-transcriptase buffer (500 mM Tris-HCl, pH 7.5, 400 mM KCl, 30 mM MgCl<sub>2</sub>), 1 µl 5 mg/ml bovine serum albumin, 5 µl 100 mM dithiothreitol, 5 µl 10 X dNTPs (10 mM of each dNTP in H<sub>2</sub>O), 3 µl H<sub>2</sub>O, 2 µl RNasin (80 units), and 2 µl reverse transcriptase (400 units) are added and the reaction is incubated at 37°C for 30 minutes. To stop the reaction, 5 µl of 3 M sodium acetate, pH 5, and 150 µl absolute ethanol are added. The tube is left at -20°C for 30 minutes, the precipitate is collected by centrifugation, washed with 80% ethanol and allowed to air-dry. The precipitate is resuspended in 10-20 µl of loading dye (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 1 mM EDTA) and the extension products are separated on a 6% sequencing gel (Maniatis, T. *et al.*, supra). Extension products are visualized by autoradiography.

### **Example (vii): S1 Nuclease Mapping**

The plasmid pBS-PR1013Cla is digested with SfaNI, dephosphorylated with calf intestinal phosphatase and kinased with <sup>32</sup>P-ATP. Following phenol extraction and ethanol precipitation, the DNA is digested with BstEII and the 300 bp fragment from 750 to 1035 of Figure 1 is isolated after electrophoresis on a low gelling temperature agarose gel. The probe is resuspended in formamide hybridization buffer (Berk, A.J. *et al.*, Cell 12, 721

- 28 -

(1977)) at a concentration of about 2 nM. The specific activity of the probe is about  $5 \times 10^6$  Cpm/pmol.

Lyophilized, total RNA (50  $\mu$ g) is dissolved in 30  $\mu$ l of the probe solution, and the tubes are heated to 65°C for 10 minutes, then allowed to hybridize overnight at 48°C. S1 nuclease treatment and gel electrophoresis are essentially as described, using an S1 nuclease concentration of 400 units/ml and an incubation temperature of 30°C. The appropriate S1 nuclease concentration is determined in pilot experiments.

**Example (viii): Mapping the Transcriptional Start Site**

The transcriptional start site for the PR-1a gene is determined by a combination of S1 nuclease mapping and primer extension analysis. An autoradiogram of a primer extension experiment using either RNA isolated from TMV-infected leaves or an mpl9 subclone of the XhoI-PstI fragment as a template and a 17 base oligonucleotide complementary to positions 1025 to 1042 of the PR-1a sequence as a specific primer is examined. The primer itself is labeled at the 5' phosphate, therefore the size of the extension product will be identical to the size of the corresponding band in the sequencing lanes. The appearance of two strong bands corresponding to positions 902 and 903 and a weak band at position 901 of the genomic clone suggests transcription initiating at either of these positions. However, primer extension analysis alone cannot be used to identify the 5' end of a mRNA. For instance, the mRNA may contain a 5' end that has been spliced from an upstream location.

To determine conclusively the 5' end, high resolution S1 nuclease mapping is used in conjunction with primer extension. An SfaNI fragment is labeled at the 5' end and digested with BstEII to yield a strand specific probe extending from position 759 to 1040. This probe is used to map the 5' end of PR-1a transcripts in RNA isolated from TMV-infected tobacco leaves. A major band of 137-2 bases is found which corresponds to positions 901 to 905 of the genomic clone. In high resolution S1 experiments, where the digestion products are electrophoresed along with a size standard of sequencing reactions performed on the probe, three bands are visualized corresponding to positions 901, 902 and 903. These results confirm the primer extension analysis and place the 5' end of the PR-1 mRNA at either position 901, 902 or 903. With regard to transcription initiation, one possible interpretation of these results is that RNA polymerase begins transcription at either base 901, 902 or 903 with more or less equal probability. However, since eukaryotic

transcription favors initiation at an A, a more likely explanation for the apparent multiple 5' ends is that the PR-1a mRNA begins at position 903 (an A) and the PR-1b and -1c mRNAs begin each at one of the other positions on their corresponding genes.

***Example (ix): General Techniques for Peptide Generation, Purification, and Automated Sequencing***

The proteins relevant to these examples are isolated, purified and sequenced in some cases, for the first time and in accordance with literature procedures in other, for the purpose of allowing the isolation of the corresponding cDNA's and ultimately for confirming the identities of their cDNA's and chemically inducible genes.

**A. Reduction and Alkylation**

Purified, lyophilized protein is dissolved in 6 M guanidine-HCl containing 1 M Tris-HCl, pH 8.6, 10 mM EDTA. Dithiothreitol is added to 20 mM and 4-vinylpyridine is added to a final concentration of 50 mM. The sample is then incubated for 1.5 hours under nitrogen. The pyridylethylated material is desalted on HPLC using an Aquapore phenyl column (2.1 x 10 cm, Brownlee). The column is eluted with a linear, 5-80% gradient of acetonitrile/isopropanol (1:1) in 0.1% trifluoroacetic acid (TFA).

**B. Cyanogen Bromide Cleavage and Removal of Pyroglutamate**

Cyanogen bromide cleavage is performed in situ according to Simpson, R.J. *et al.*, *Biochem. Intl.* 8: 787 (1984) Digestion of PR-1 protein with pyroglutamate aminopeptidase (Boehringer Mannheim) is carried out according to Allen, G., *Plant Sci. Lett.* 26: 173 (1982).

**C. LysC digestion**

Protein is digested with endoproteinase Lys-C (Boehringer Mannheim) in 0.1 M Tris-HCl, pH 8.5, for 24 hours at room temperature using an enzyme:substrate ratio of 1:10. Resulting peptides are isolated by HPLC using an Aquapore C-8 column (1 x 22 cm, Brownlee) eluted with a linear acetonitrile/isopropanol (1:1 ratio) gradient (0 to 60%) in 0.1% TFA.

- 30 -

#### D. Trypsin Digestion

Digestion with trypsin (Cooper) is performed in 0.1 M ammonium bicarbonate, pH 8.2, containing 0.1 M calcium chloride for five hours at 37°C using an enzyme:substrate ratio of 1:100. Peptides generated are separated on HPLC using the same conditions as with the Lys-C peptides or performed in 0.1 M Tris-HCl pH 8.5 for 24 hours at 37°C using an enzyme to substrate ratio of 1:50. Peptides are isolated by HPLC using a Vydac C-18 column (2.1 x 150 mm) with a linear 0 to 60% acetonitrile:isopropanol (1:1) gradient in 0.1 % TFA.

#### E. Sequencing

Automated Edman degradations are performed with an Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin (PTH) amino acids are identified using an Applied Biosystems 120A PTH analyzer.

#### ***Example 1A: Cloning of cDNAs corresponding to SAR CHX-independent genes from tobacco***

A number of cDNAs were cloned by differential screening from cDNA prepared from induced and non-induced tissue. The induced cDNA was prepared from tobacco leaves which had been pre-treated with methyl benzo-1,2,3-thiadiazole-7-carboxylate (BTH), whereas the non-induced cDNA was prepared from tissue which had not been pre-treated with BTH. cDNA libraries were prepared in  $\lambda$ ZAP II (STRATAGENE). A standard differential screening technique was used. Plaques carrying induced cDNA were plated at low density and transferred to two sets of hybridization filters. Known SAR gene sequences were hybridized to the first filter and uninduced cDNA to the second. The second filter was then stripped and hybridised with induced cDNA. Plaques which hybridized with the induced cDNA probe, but not with the uninduced cDNA probe or with known SAR gene sequences were potential novel SAR genes and were picked directly for further analysis. Plating had been at a plaque density which was sufficiently low to enable these plaques to be picked as nearly pure plaques. Multiple candidates for each plaque were *in vivo* excised, according to the manufacturer's recommended conditions, for further screening by Northern hybridization to RNA isolated from either untreated or BTH-treated tobacco plants. Individual clones chosen from the secondary screen were further analyzed by Northern



- 31 -

hybridisation to RNA isolated from tobacco plants which had been pre-treated with salicylic acid (3 mM), INA (1 mM), and BTH (1 mg/ml), all in the presence or absence of cycloheximide (CHX; 1 mg/ml). Inducer pre-treatments were done at 2 h, one day, and eight days before the isolation of RNA, whereas CHX treatment was done at one day before isolation of RNA. Three cDNAs were found induced in a protein synthesis independent fashion. The genes corresponding to these cDNAs have been designated p66B1 (SEQ ID NO. 14), p11.30.13 (SEQ ID NO. 11), and p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) and are likely signal transducers of the SAR response. Gene p1.4.3 has previously been disclosed as a thioredoxin (Brugidou *et al.*, *Mol. Gen. Genet.* 238: 285-293 (1993)). However, this is the first disclosure of the gene's likely involvement in the systemic acquired response. Three cDNAs were found to be expressed in a protein synthesis dependent fashion. These were designated p1.1.1, p11.31.4, and p14.22.3 and are listed as SEQ. ID Nos. 9, 10, and 15, respectively.

***Example 1B: Cloning of cDNAs corresponding to SAR CHX-independent genes from Arabidopsis using INA for induction***

Total RNA was isolated from the following Arabidopsis lines: (1) untreated, (2) INA treated (0.25 mg/ml), (3) CHX treated (1 mg/ml), and (4) INA + CHX treated. Treatments were made 1 day before RNA isolation. RNA thus isolated was subjected to "differential display" using the protocol described by Liang and Pardee, *Science* 257: 967-971 (1992). Amplified fragments which were found in both the INA as well as the INA + CHX treated RNA samples were gel-purified and used as probes on Northern blots carrying similarly induced RNA samples. Fragments for which Northern hybridization confirmed the induction profile apparent from differential display were subcloned into a plasmid vector. Using the cloned fragment it was possible to isolate near full-length cDNAs from a cDNA library produced by BTH induction:

Mature *Arabidopsis thaliana* ecotype Columbia (Lehle Seeds, Tucson, AZ) plants are sprayed with a 0.5 mg/ml suspension of a wettable powder formulation of methylbenzo-1,2,3-thiadiazole-7-carboxylate consisting of 25% active ingredient. Seven days later, the leaf tissue is harvested and frozen in liquid N<sub>2</sub>. Total RNA is isolated as described in Example (vi) and Poly (A)<sup>+</sup> RNA is isolated using a Poly (A) Quik<sup>TM</sup> mRNA isolation kit from Stratagene (La Jolla, CA). This Poly (A)<sup>+</sup> RNA is then used to make a cDNA library in the

- 32 -

uni-zap<sup>TM</sup> XR vector (Stratagene) using a ZAP-cDNA<sup>TM</sup> Gigapack<sup>R</sup> II Gold cloning kit from Stratagene. A portion of the cDNA library is amplified as described in the Stratagene kit.

The cDNA pDPA2 was cloned using this technique and is induced by the SAR response in a protein synthesis independent fashion. Its sequence is listed in Seq. ID No. 16.

**Example 1C: Cloning of cDNAs corresponding to SAR CHX-independent genes from Arabidopsis using BTH for induction**

RNA isolated from (1) untreated, (2) BTH treated, and (3) BTH + CHX treated Arabidopsis lines was subjected to "differential display" using the protocol described by Liang and Pardee, *Science* 257: 967-971 (1992) and as described in example 1B above. Amplified fragments which were found in both the BTH as well as the BTH + CHX treated RNA samples were gel-purified and used as probes on Northern blots carrying similarly induced RNA samples. Fragments for which Northern hybridization confirmed the induction profile apparent from differential display were subcloned into a plasmid vector. The following clones induced in a protein synthesis-independent manner were obtained:

- PSI-1 (SEQ ID NO. 33): cDNA homologous to EMBL Accession #Z26429, an *A. thaliana* transcribed sequence (clone FAFC80-1; 5' end and actin depolymerizing factor from *L. longiflorum*), and EMBL Accession #Z14110, an *L. longiflorum* mRNA for actin depolymerizing factor
- PSI-2 (SEQ ID NO. 34): cDNA with no known homology
- PSI-3 (SEQ ID NO. 35): cDNA homologous to EMBL Accession # T20772, *A. thaliana* cDNA 92H8T7, and EMBL Accession #T04101, *A. thaliana* cDNA clone SCG7T7P
- PSI-4 (SEQ ID NO. 36): cDNA homologous to EMBL Accession #D22118, a partial Rice cDNA sequence
- PSI-5 (SEQ ID NO. 37): cDNA with no known homology

**Example 2A: Cloning of Class IV Chitinase cDNAs**

To clone recombinant or chimaeric DNA molecules encoding class IV chitinase class specific degenerate oligonucleotides were designed from areas of homology between bean PR4 chitinase (Margis-Pinheiro *et al.*, *Plant Mol. Biol.* 17: 243-253 (1991)) and sugar beet chitinase 4 (Mikkelsen *et al.*, in "Advances in Chitin and Chitosan, ed. by Brine *et al.*, pub. by Elsevier, Amsterdam (1992)): oligonucleotides were designed degenerate for the peptide

sequences HFCYIEE (forward, spanning nucleotides 406-426; SEQ ID NO. 27), and IRAINNG (reverse, spanning nucleotides 705 to 675; SEQ ID NO. 28). DNA was extracted from two-week old plants of *Arabidopsis thaliana* ecotype Landsberg and amplified using a Perkin-Elmer thermal cycler 480 at the following cycle settings: 94°C for 5 minutes; 35 cycles at 94°C, 1 minute, 43°C or 45°C, 1 minute, and 72°C, 2 minutes; followed by 5 minutes at 72°C. The amplified fragment was gel purified, collected by centrifugation through Whatman paper, ethanol precipitated, resuspended in TE, digested with BamHI and NsiI and subcloned into pTZ18U (Pharmacia). Four clones of the fragment were sequenced; they differed only within the oligo-derived sequence as could be expected from amplification with degenerate oligonucleotides.

Two different cDNAs were isolated simultaneously by screening a leaf tissue cDNA library (Uknes *et al.*, *Plant Cell* 4: 645-656 (1992)) at high stringency with the PCR amplified genomic fragment described above. Duplicate plaque lifts were taken with nitrocellulose filters (Schleicher & Schuell, Keene, NH) (Ausubel *et al.*, in "Current Protocols in Molecular Biology, pub. by J. Wiley & Sons, New York (1987)). Probes were labelled by random priming (using the labeling system supplied by Gibco BRL, Gaithersburg, MD). Hybridization and washing were done at 65°C according to Church and Gilbert, *Proc. Natl. Acad. Sci.* 81: 1991-1995 (1984). Positive plaques were purified and plasmids containing the cDNA inserts were *in vivo* excised for DNA sequence determination. Of the six positive clones two contained an insert with structural homology to previously characterized class IV chitinases and were designated class IV chitinase type A and four clones were divergent in that they lacked the class IV chitinase hevein domain; these were designated class IV chitinase type B. As none of the cDNA inserts was full-length, an additional 29 bp of class IV chitinase type A and 17 bp of class IV chitinase type B, both containing a methionine initiation codon were amplified from ethephon-induced RNA using the 5' RACE system for rapid amplification of cDNA ends (Gibco BRL, Gaithersburg, MD). Sequence comparisons were performed using the GAP and PILEUP features of the Genetics Computer Group software (Genetics Computer Group, Madison, WI).

Class IV chitinase type A is a 1079 base pair cDNA with an open reading frame of 264 amino acids containing the characteristic cysteine-rich hevein and chitinolytic domains and the three short deletions typically found in class IV chitinases (see SEQ ID No. 1). The cDNA for class IV chitinase type B is 952 base pairs in length and encodes a protein of 214 amino acids which lacks a hevein domain and contains a fourth deletion (see SEQ ID No.

2). The cDNAs are 71% identical overall and 80% identical over coding sequence. The predicted protein encoded by Arabidopsis class IV chitinase type A is 89% homologous to the basic Brassica napus (rapeseed) class IV chitinase, 61% homologous to basic Beta vulgaris (sugar beet) class IV chitinase, 57% homologous to basic Zea mays class IV chitinase B, 58% homologous to acidic Phaseolus vulgaris (bean) PR4 class IV chitinase, and 55% homologous to acidic Dioscorea japonica (yam) class IV chitinase. It is 42% homologous to Arabidopsis thaliana basic class I chitinase.

The predicted mature protein encoded by Arabidopsis class IV chitinase type A has a molecular weight of 25695 D and a pI of 7.8; whereas the protein encoded by Arabidopsis class IV chitinase type B has a molecular weight of 20553 D and a pI of 10 assuming the removal of a signal peptide based on homology to tobacco PR-Q.

Northern analysis showed that both chitinases were induced by TCV infection, confirming their classification as PR-proteins.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

#### ***Example 2B: Isolation of Chemically Induced Wheat cDNAs Using the Method of Differential Plaque Filter Hybridization***

Recombinant or chimaeric DNA molecules comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a plant were obtained in the following way:

Samples of winter wheat (cultivar Kanzler) were harvested 2-3 days after treatment with either water or 200 ppm of the plant activator compound benzo-1,2,3-thiodiazole-carboxylic acid. Total RNA was prepared from frozen tissue samples using a standard phenol extraction/LiCl precipitation procedure (Lagrimini *et al.*, *Proc. Natl. Acad. Sci.* **84**: 7542-7546 (1987)). PolyA (+) RNA was purified from total RNA using the Poly(A) Quik mRNA purification kit (Stratagene Cloning Systems, LaJolla, CA). A bacteriophage lambda ZAP II cDNA library was prepared from the benzo-1,2,3-thio-diazolecarboxylic acid treated polyA(+) sample using the Uni-Zap XR Gigapack II Gold cloning kit (Stratagene) as described by the manufacturer. The phage library was plated at a density of approximately 5000 plaques on a 10 cm petri dish and grown for 6-8 hours at 37°C. Duplicate filter lifts of

- 35 -

the plaques were made using nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Labelled first strand cDNA probes were prepared from polyA of both the water control and the benzo-1,2,3-thiodiazolecarboxylic acid-treated samples using  $^{32}\text{P}$ -dCTP and the AMV reverse transcriptase (GibcoBRL, Gaithersburg, MD) under the manufacturer's conditions. Each probe was hybridized ( $>106$  cpm/ml) with one set of the duplicate lifts overnight at  $65^{\circ}\text{C}$ . Hybridization and wash conditions were as described in Church and Gilbert, *Proc. Natl. Acad. Sci.* 81: 1991-1995 (1984). Hybridization was detected by autoradiography.

Plaques appearing to hybridize preferentially to the chemically treated cDNA were purified and their cDNA inserts were amplified using the GeneAmp Polymerase Chain Reaction (PCR) kit (Perkin Elmer, Norwalk, CT) and primers homologous to the flanking lambda Zap II sequences. The amplified inserts were excised from a low melting temperature SeaPlaque GTG agarose gel (FMC BioProducts, Rockland, ME) and labelled using  $^{32}\text{P}$ -dCTP and the Random Primers DNA Labeling System (Gibco BRL). These probes were hybridized with total RNA blots (Ausubel *et al.*, in "Current Protocols in Molecular Biology, pub. by J. Wiley & Sons, New York (1987)) of control and benzo-1,2,3-thiodiazolecarboxylic acid treated RNAs to verify that they contained chemically induced cDNAs.

The induced clones were *in vivo* excised into pBluescript plasmids according to the manufacturer's instructions (Stratagene) and plasmid DNAs were purified using Magic Miniprep columns (Promega Biotech, Madison, WI). The cDNA sequences were determined by the chain termination method using dideoxy terminators labelled with fluorescent dyes (Applied Biosystems, Inc., Foster City, CA). The DNA and the predicted amino acid sequences were compared to available databases using the GAP (Deveraux *et al.*, *Nucl. Acids Res.* 12: 387-395 (1984)) and the BLAST (Altschul *et al.*, *J. Mol. Biol.* 215: 403-410 (1990)) programs.

The nucleotide sequence of one apparently full length induced clone is set forth in SEQ ID No. 3. This clone, denoted WCI-1 (Wheat Chemical Induction), was found to share limited homology with two rice cDNAs, one expressed specifically in the shoot apical meristem (De Pater and Schilperoort, *Plant Mol. Biol.* 18: 161-164 (1992)), the other inducible by salt stress (Claes *et al.*, *Plant Cell* 2: 19-27 (1990)). The function of these proteins is unknown.

The predicted amino acid sequence of a second highly induced cDNA, WCI-2, clearly identified it as an isozyme of wheat lipoxygenase, based on its homology to other

- 36 -

plant lipoxygenases in the database. The DNA sequence of this cDNA is shown in SEQ ID No. 4.

A third class of induced cDNA (WCI-3) was isolated which to date shows no significant homology to sequences in the databases. The DNA sequence of this apparently full-length clone is set forth in SEQ ID No. 6.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 2C: Isolation of Wheat cDNAs Specifically Induced by Treatment With benzo-1,2,3-thiodiazolecarboxylic acid, Using the Method of Differential cDNA Display***

The total and polyA(+) samples described in Example (vi) were used for PCR differential display of the mRNA from water and benzo-1,2,3-thiodiazolecarboxylic acid treated wheat tissue essentially as described in Liang and Pardee, *Science* 257:967-971 (1992). Amplified cDNA fragments that appeared to be present only in the chemically treated sample were excised from the dried sequencing gel and electroeluted using a Centrilotur device and Centricon-30 Microconcentrators (Amicon, Beverly, MA). The purified fragments were PCR amplified using primers that consisted of the original differential display 10-mers plus an additional 10 bases of unique sequence added to their 5' ends. After 8 PCR cycles at low annealing temperature (42-45°C), the annealing temperature was raised to 60°C for an additional 30 cycles. Essentially 100% of the fragments could then be visualized by EtBr staining and were excised from a SeaPlaque GTG agarose gel (FMC).

These gel fragments were labelled and used to probe RNA blots as previously described. Fragments that hybridized only with chemically treated RNA were TA-cloned into the plasmid vector pCR II using a TA Cloning Kit (Invitrogen Corporation, San Diego, CA). Inserts from the plasmids were screened again against control/chemical RNA blots to verify that the inducible gene fragment had been subcloned. Induced fragments were then labelled by random priming and hybridized against filter lifts of the chemically induced cDNA library as described in Example 2A. Hybridizing plaques were purified, sequenced, and analyzed as previously described in order to obtain full length clones corresponding to the

- 37 -

original small (200-400 bp) fragments, and to identify the induced gene product where possible.

The nucleotide sequence of one clone obtained by this procedure is set forth in SEQ ID No. 7. This clone, WCI-4, has some homology to known thiol protease sequences from a variety of sources and may therefore be a thiol protease.

Partial sequences of an additional induced gene that was isolated using differential display is set forth in SEQ ID No. 8. This fragment shows no database homology and has been designated WCI-5.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 3A: Preparation of cDNA Library From TMV-Infected Tobacco Leaves.***

*Nicotiana tabacum* cv. Xanthi-nc leaves are infected with tobacco mosaic virus and harvested five days post-infection. Total RNA is prepared as described above and poly A+ RNA is isolated using standard techniques. A cDNA library is constructed using this poly A+ RNA in the Lambda onC cloning vector (Stratagene) essentially as described (Gubler, U. and Hoffman, B. J., *Gene* 25: 263 (1983)).

***Example 3B: Isolation of cDNA Clones Encoding Chitinase/Lysozymes***

To clone recombinant or chimaeric DNA molecules encoding chitinase/lysozyme about 300,000 plaques of the TMV-infected tobacco cDNA library described in Example 3A are screened using a labeled cDNA probe encoding the cucumber chitinase/lysozyme cDNA and washing filters at 50°C in 0.125 mM NaCl, 1% SDS, 40 mM sodium phosphate (pH 7.2), 1 mM EDTA. Positive plaques are purified and the DNA sequence of two clones, named pBSCL2 and pBSTCL226 are determined. These are presented in SEQ ID Nos. 20 and 21, respectively. The proteins encoded in the clones of these sequences are determined to be chitinase/lysozymes based on structural homology to the cucumber chitinase/lysozyme.

In addition, a protein is purified from intercellular fluid of TMV-infected tobacco. Peptides are generated and sequenced as described in Example (ix)

- 38 -

The protein encoded by pBSTCL226, corresponding to an acidic isoform of chitinase/lysozyme, was found to match the deduced peptide sequences exactly.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 3C: Analysis of Seed Lines Derived From Transformation of Tobacco with the Basic and Acidic Class III Tobacco Chitinase Genes***

Binary constructions carrying the tobacco basic and acidic class III chitinases were transformed into *Nicotiana tabacum* cv *Xanthi-nc*. Leaf tissue samples were taken from T1 plants and assayed for expression of either transgene protein (by Western analysis) or transgene RNA (by Northern analysis). T1 plants found to express the transgene at high levels were advanced to T3 seed lines as described above.

***Example 3D: Analysis of Transgenic Plants Expressing Class III Chitinase for Pest Resistance***

Transgenic plant lines expressing one or more class III chitinase genes are assessed for resistance to numerous pests. Approximately six plants of each line are tested. Pests at the appropriate stage of their growth cycle (such as larvae) are introduced at the appropriate stage of plant development. Plants are later assayed for % of leaf or tissue area eaten, % of introduced larvae surviving, and the weight of surviving larvae.

***Example 3E: Analysis of Transgenic Tobacco Plants Expressing Class III Chitinase for Pest Resistance***

Transgenic tobacco plants expressing class III chitinase are assessed for resistance to numerous pests. Those tested include *Spodoptera exigua* (beet armyworm), the green peach tobacco aphid, *Manduca sexta*, and various nematodes, weevils, mites, and other pests.



**Example 3F: Analysis of Transgenic Plants Expressing Basic Class III Chitinase for Resistance to *Heliothis virescens***

Transgenic tobacco lines expressing the tobacco basic class III chitinase gene were assessed for resistance to the insect *Heliothis virescens*. Eight plants of each line were tested 50 mm leaf discs were cut from the youngest leaves of transgenic plants approximately six weeks after germination and 3 larvae (2nd stage) were allowed to feed on each disc for 3-7 days. After 3-7 days the leaf discs were assessed for area eaten, % of larval survivors and weight (in mg) of larval survivors. Results of three separate experiments are shown in the tables provided below.

The results show elevated resistance to *Heliothis* in basic class III chitinase overexpressing plants when compared to non-transformed control lines *Heliothis virescens* (the tobacco budworm) causes considerable damage in tobacco crops and is particularly recalcitrant to control using pesticides as the larvae burrow deep within the plant. Furthermore, other *Heliothis* species cause similar damage to cotton and other crops.

- 40 -

	(1)	%LEAF EATEN	%SURV IVORS	WT (mg) LARVAE	(2)	%LEAF EATEN	%SURV IVORS	WT(mg) LARVAE
Line								
Control		72	100	23		77	100	26
3505 C-15-5		45	92	14		65	96	19
3505 C-5-3		55	96	17		60	92	23
3505 C-4-2		57	92	19		60	100	20
3505 C-12-8		-	-	-		44	87	15

	(3)	%LEAF EATEN	%SURV IVORS	WT (mg) LARVAE
Line				
Control		65	100	53
3505 C-12-8		59	100	34

A summary of the two experiments detailed above is given below:

(1) Line:	Control	3505 C-15-5	3505 C-5-3	3505 C-4-2
Resistance Index	0	4	2.5	2

(2) Line:	Control	3505 C-15-5	3505 C-5-3	3505 C-4-2	3505 C-12-8
Resistance Index	0	2	3	3	4

(3) Line:	Control	3505 C-12-8
Resistance Index	0	3

- 41 -

## Resistance Index:

- S = significantly more susceptible than control plants (i.e. wild-type)
- (S) = more susceptible than control plants, but not significantly in all evaluations
- 0 = like wild type plants
- 1 = some signs of resistance but not significantly different to controls
- 2 = identified resistance, significant only in some evaluations
- 3 = resistance with small significant differences in all evaluations
- 4 = high resistance, but symptoms or damage can still be observed
- 5 = total resistance. No symptoms or damage.

**Example 4A: Isolation of a Genomic Clone for Arabidopsis PR-1**

The *Arabidopsis* PR-1 cDNA cloned in pAPR1C-1 (sequence SEQ ID No. 17) was used as a hybridization probe in screening an *Arabidopsis*  $\lambda$ EMBL 3 genomic library (purchased from Clontech). Four hybridizing plaques were plaque purified using conventional techniques and  $\lambda$ DNA was isolated from each one with lmbdasorb (Promega). The  $\lambda$ DNA thus isolated was digested with XhoI, electrophoresed, transferred to hybridization membrane for hybridization with the PR-1 cDNA. A fragment of 7 kb was found to hybridize to the cDNA. DNA from one of the four purified plaques was then redigested with XhoI and ligated into the XhoI site of pBluescript (Stratagene). A colony carrying a recombinant or chimaeric DNA molecule comprising the promoter region of the *Arabidopsis* PR-1 gene was identified by probing with oligonucleotide DC21 from the PR-1 coding sequence (position +110 to +84) and the plasmid contained therein was designated pAtPR1-P and deposited January 5, 1994 with the the Agricultural Research Culture Collection, International Depositing Authority, 1815 N. University Street, Peoria, Illinois 61604 under the accession no. NRRL B-21169. Restriction analysis identified the 7 kb XhoI fragment as extending 4.2 kb upstream of the ATG of the PR1 gene.

**Example 4B: Fusion of the Arabidopsis PR-1 Promoter to the Firefly Luciferase Gene**

Plasmid pDO432 containing a gene encoding luciferase (LUC) from firefly was received from Dr David Ow (University of California, San Diego; see Ow *et al.*, 1986; Science 234: 856). The LUC gene was excised from pDO432 by digestion with XbaI (at position +45 relative to the ATG) and SstI (approximately 1.8 kb downstream of the ATG and outside the LUC coding region). Additionally, an EcoRI-XbaI promoter fragment was

- 42 -

excised from pAtPR1-P; this fragment was 1.4 kb in size and delineated by an XbaI site 2.8 kb upstream of the PR-1 ATG and an EcoRI site in the pBluescript polylinker distal to the 5' end of the cloned promoter fragment (at -4.2 kb relative to the ATG). These two fragments were cloned by threeway ligation into EcoRI/SacI cleaved pBluescript thus orienting the LUC gene adjacent to the upstream PR-1 promoter fragment (pAtPR1-Q).

Subsequently, pAtPR1-P was cleaved with XbaI (at the -2.8 kb position and within the pBluescript polylinker) and religated to generate a PR-1 genomic construct without the upstream 1.4 kb promoter fragment and which thus ended 2.8 kb upstream of the PR-1 ATG (pAtPR1-R). This plasmid was used as a template in PCR with a left-to-right "topstrand" primer extending from positions -237 to -214 (DC39) upstream of the PR-1 ATG (oligo A) and a right-to-left "bottomstrand" primer comprising 15 bp of LUC coding sequence extending up to the LUC ATG and a further 19 bp of PR-1 sequence extending from the ATG into the PR-1 untranslated leader (oligo B: sequence: TTT GGC GTC TTC CAT TTT TCT AAG TTG ATA ATG G; SEQ ID No. 18). This PCR reaction was undertaken for five cycles at 94°C (30 s), 40°C (60 s), and 72°C (30 s) followed by 25 cycles at 94°C (30 s), 55°C (60 s) and 72°C (30 s) and this generated a product of 245 bp through annealing of the homologous PR-1 sequences; the fragment included a BglII site at its left end from the PR-1 promoter. A second PCR reaction was done using plasmid pDO432 as a template and using a left-to-right "topstrand" oligonucleotide which comprised 15 bp of PR-1 untranslated leader up to the PR-1 ATG and a further 12 bp of LUC sequence from the ATG into the LUC coding sequence (oligo C: sequence: TAT CAA CTT AGA AAA ATG GAA GAC GCC AAA; SEQ ID No 19) and a right-to-left "bottom strand" oligonucleotide extending from positions 332 to 312 (DC53) into the LUC coding sequence (oligo D). This PCR reaction was done under the same conditions as the one described above and generated a fragment of approximately 300 bp through annealing of the homologous LUC sequences; this fragment included a PstI site at its right end, derived from the LUC sequence amplified.

The two PCR fragments generated above were gel purified using standard procedures to remove oligonucleotides and were then themselves mixed in a further PCR reaction ("inside-outside PCR") with oligonucleotides A and D as primers. Conditions for this reaction were the same as described above. The amplified fragment was a fusion of the PR-1 promoter fragment from the first PCR reaction described above and the LUC 5' coding sequence from the second PCR reaction described above and had a BglII site at its left end and a PstI site at its right end. The fragment was gel purified and cleaved with BglII and PstI

- 43 -

to yield a product of 545 bp in size which was cloned into pAtPR1-R which had previously been cleaved with the same enzymes. Cleavage of the resultant plasmid (pAtPR1-S) with XbaI released a PR-1 promoter fragment extending from -2.8 kb to the XbaI site downstream of the LUC ATG, the fusion point between the PR-1 promoter and the LUC coding sequence being at the ATG. This fragment was cloned into XbaI cleaved pAtPR1-Q regenerating the full-length PR-1 promoter (4.2 kb) in operational fusion to LUC (pAtPR1-R).

**Example 4C: Transfer of the *Arabidopsis* PR-1 Promoter - Firefly Luciferase Gene Fusion to pCIB200**

TJS75Kan is first created by digestion of pTJS75 (Schmidhauser and Helinski, *J. Bacteriol.* 164: 446-455 (1985)) with NarI to excise the tetracycline gene, followed by insertion of an AccI fragment from pUC4K (Messing, J. and Vierra, J., *Gene* 19: 259-268 (1982)) carrying a NptII gene. pCIB 200 is then made by ligating XhoI linkers to the EcoRV fragment of pCIB7 (containing the left and right T-DNA borders, a plant selectable nos/nptII chimeric gene and the pUC polylinker, Rothstein, S.J. *et al.*, *Gene* 53: 153-161 (1987)) and cloning XhoI digested fragment into Sall digested TJS75Kan.

pAtPR1-R was cleaved with XhoI and SacI and transferred to Sall/SacI cleaved pCIB200 to create a binary vector construction (pAtPR1-S) suitable for *Arabidopsis* transformation. pAtPR1-S was then transferred to *Agrobacterium tumefaciens* strain A136/pCIB542 for transfer to *Arabidopsis* Dijon-0 using the method described by Wen-jun and Forde, *Nucl. Acids Res.* 17: 8385-8386 (1989). T2 or T3 lines carrying the PR1-LUC transgene in the homozygous state were generated for chemical induction analysis.

**Example 4D: Chemical Induction of the *Arabidopsis* PR-1 Promoter**

Leaves of transgenic *Arabidopsis* lines carrying the PR-1 promoter-LUC gene fusion were treated by spraying with 0.15 µg/ml INA (isonicotinic acid) for 48 h. Five days later, sprayed leaves were analyzed for luciferase activity using the Promega Luciferase Assay System (Cat. # E 1500). Transgenic lines showed 144-fold induction over controls. In other assays, luciferase activity was measured by imaging lines for bioluminescence using intensified cameras (VIM) and photon-counting image processors (ARGUS-50 or ARGUS-100) from Hamamatsu Photonic Systems (Bridgewater, NJ). Leaves were sprayed with INA and subsequently with a 5 mM solution of D-luciferin (Analytical Bioluminescence

- 44 -

Laboratories, San Diego, CA) 24 h before and then immediately before imaging. Transgenic plants carrying the PR1-LUC fusion had strongly induced bioluminescence when compared to water-treated controls.

**Example 5: Isolation of the Maize PR-1 and Thaumatin-like cDNAs**

The maize PR-1-like cDNA PR-1mz was isolated by screening a BTH-induced cDNA library of maize with a probe matching to the PR-1 barley clone HVPR1BR (EMBL data base Accession # X74940). The DNA sequence of the gene and the deduced amino acid sequence are shown in SEQ ID NOs. 29 and 30, respectively.

The maize thaumatin-like cDNA PR-5mz was isolated by screening a BTH-induced cDNA library of maize with a probe matching to a known rice thaumatin clone (Reimann and Dudler, Plant Physiol. 101:1113-1114). The DNA sequence of the gene and the deduced amino acid sequence are shown in SEQ ID NOs. 31 and 32, respectively.

**Example 6A: Transformation of Disease Resistance Genes into Maize**

Ciba maize inbred line CGA00526 was transformed (Koziel, et al., Biotechnology 11:194-200, 1993) with tobacco *PR-1a*, tobacco *SAR 8.2*, and wheat *Thionin* disease resistance genes. Constructs used for transformation include the chimeric plasmid pUBA (Toki et al, Plant Physiol. 100: 1503-1507, 1992) containing the promoter, first exon, and first intron of the maize ubiquitin gene (*Ubi-1*), and the coding sequence of the *bar* gene conferring resistance to phosphinothricin (Thompson et al., EMBO J. 6: 2519-2523, 1987). For cointegration purposes, the coding sequence of either the tobacco *PR1a* gene (Alexander et al, Proc. Natl. Acad. Sci. USA 90: 7327-7331, 1993), the tobacco *SAR8.2* gene (Alexander et al., 1992, Mol. Plant-Microbe Inter. 5:513-515), or the thionin gene from wheat (Rodriguez-Palenzuela et al., 1988, Gene 70:271-281), each under the control of said ubiquitin promoter were constructed into this plasmid resulting in three separate constructs for transformation. PCR techniques were used to obtain suitable restriction sites around the coding sequences of *PR1a*, *SAR8.2*, and wheat thionin, respectively. Maize tissue was bombarded with each plasmid separately or cotransformed with the *PR-1a* and *SAR8.2* constructs. Expression of the tobacco disease resistance genes *PR-1a* and *SAR 8.2*, a combination of *PR-1a* and *SAR 8.2*, and the wheat *Thionin* gene (Rodriguez-Palenzuela et al., Gene. 70:271-281, 1988) was confirmed by Northern blots.

**Example 6B: Transformation of Disease Resistance Genes into Wheat**

Immature embryos of spring wheat were isolated aseptically approximately 2 weeks after pollination, and plated scutellum side uppermost on MS based medium (Murashige and Skoog, 1962 *Physiol. Plant* 15:473-439) supplemented with 2,4-D, glutamine and asparagine. Four to six hours prior to transformation embryos were transferred to medium also containing 0.25M mannitol. Constructs used for transformation included the chimeric plasmid pUBA (Toki et al, *Plant Physiol.* 100:1503-1506, 1992) containing the promoter, first exon and first intron of the maize ubiquitin gene (Ubi-1), and the coding sequence for the bar gene conferring resistance to phosphinothricin (Thomson et al, *EMBO J.* 6:2519-2523, 1987). This construct was co-transformed with a single plasmid construct containing the coding sequences for the two disease resistance genes PR1a (Alexander et al, *Proc. Natl. Acad. Sci. USA* 90:7327-7331, 1993) and the SAR 8.2 (Alexander et al, *Mol. Plant-Microbe Inter.* 6:513-515, 1992) from tobacco, expression of both sequences being driven by the double 35S promoter. For cloning purposes PCR techniques were used to obtain suitable restriction sites around the coding sequences of PR1a and SAR 8.2, respectively,. Plasmid DNA was precipitated onto gold particles as described in the Dupont Biolistic manual. Embryos were bombarded using the PDS 1000 biolistic device (Weeks et al, *Plant Physiol* 102:1977-1084, 1993) and transferred the following day to the original medium without added mannitol. Calli which developed from the bombarded embryos were selected for a morphogenic response and regenerated on medium containing Basta. Presence of transgene fragments were confirmed by PCR. Total RNA was extracted (Lagrimini et al, 1987 *Plant Physiol* 84:438-442) and gene expression confirmed by Northern analysis. Transformed plants contained and expressed the bar gene and the PR1a gene. Some events also expressed the SAR 8.2 gene. Plants were selfed and Basta resistant progeny obtained.

**Example 6C: Alternative Methods for Introducing and Expressing More Than One Anti-Pathogenic Sequence In Plant Tissue**

In addition to the possibility of expressing more than one transgene in transgenic lines by the sexual crossing of lines which are transgenic for one gene, the skilled artisan recognizes that an equivalent way of generating lines transgenic for more than one gene is by the use of transformation plasmids which carry more than one gene. For example, the expression of two cDNAs in a transgenic line can be achieved by the transformation of the host plant with a vector carrying each cDNA under the independent regulation of two promoters *i.e.* the vector carries two expression cassettes in addition to sequences needed for antibiotic selection *in vitro*. Each expression cassette can also carry any signal

- 46 -

sequence, vacuolar targeting sequence and transcriptional terminator so desired. Vectors carrying multiple expression cassettes can be constructed for use with *Agrobacterium* transformation or direct gene transfer transformation systems.

A further method for the expression of more than one transgene in a transgenic plant line is to firstly transform with a single gene (with appropriate regulatory signals) carried on a transformation vector and subsequently transform a line selected from this transformation with a further gene (with appropriate regulatory signals) carried on a different plasmid which utilizes a different antibiotic selection system. This method is obvious to those of skill in the art.

**Example 6D: Synergistic Effect of Combined Anti-Pathogenic Sequences**

Overexpression of two or more PR proteins in a transgenic plant gives rise to a synergistic anti-pathogen effect. The table below shows data from an experiment in which control tobacco lines and tobacco lines rendered transgenic for PR-1a, SAR8.2, or PR-1a and SAR 8.2 were inoculated with the pathogen *Peronospora tabacina* 5 and 8 days after inoculation, percentage leaf area infected was assessed. The constructs used to obtain the transgenic tobacco lines are similar to the constructs described in examples 6A and 6B. Tobacco lines transgenic for both PR-1a and SAR8.2 were obtained according to example 6C. The left side of the table shows raw data from numerous lines of each phenotype, whereas the right side shows mean data for each phenotype. In addition to the mean values for percentage leaf area infected, the relative area compared as a percentage of the control (=100%) is presented. This enables a calculation of the expected value in the line expressing both PR-1a and SAR8.2 assuming that the individual components are additive in action. The observed values of 40.8 and 30.4 % are well below the expected values of 63.4 % ( $0.756 \times 0.838 \times 100$ ) and 62.4 % ( $0.694 \times 0.899 \times 100$ ) for 5 and 8 days post-inoculation. These results demonstrate that the disease resistance effects of PR-1a and SAR8.2 are synergistic in transgenic plants.



- 47 -

**Table A**

	DAYS AFTER INOCULATION			MEAN DATA DAYS AFTER INOCULATION	
	5	8		5	8
Control (L6)	36.1	42.2	<b>CONTROL</b>	40.2	49.4
Control (L18)	42.8	51.1		(100)	(100)
Control (L22)	41.7	55.0			
PR-1a (L2)	35.0	42.8	<b>PR-1a</b>	30.4	34.3
PR-1a (L7)	25.0	25.0		(75.6)	(69.4)
PR-1a (L13)	31.1	35.0			
SAR8.2 (L1)	36.7	49.4	<b>SAR8.2</b>	33.7	44.7
SAR8.2 (L8)	36.1	45.0		(83.8)	(89.9)
SAR8.2 (L11)	26.7	45.0			
SAR8.2 (L21)	35.6	46.1			
SAR8.2 (L25)	33.3	37.8			
PR-1a/SAR8.2 (L17)	16.1	15.0	<b>PR-1a/SAR8.2</b>	16.1	15.0
				(40.8)	(30.4)
<u>Expected Additive Values:</u>				<u>63.4</u>	<u>62.4</u>

**Example 7: Expression of SAR/CHX-independent genes in transgenic plants**

The cDNAs described in examples 1A and 1B can be expressed in transgenic plants using techniques well known in the art. As components of the signal transduction pathway involved in SAR, the CHX-independent genes are useful for the manipulation of the SAR response. For example, the constitutive expression of key components in the SAR transduction pathway in transgenic plants will likely lead to the generation of plants with

- 48 -

enhanced disease resistance characteristics and this will likely be achieved by the activation of components in the pathway downstream to the component being expressed transgenically and hence to the activation of anti-pathogenic end products. By way of illustration this may be achieved from the expression of the appropriate genes behind the constitutive 35S promoter cDNAs may be transferred to the vector pCGN1761 or pCGN1761/ENX which carry the double 35S CaMV promoter and the *tm1* transcriptional terminator on a pUC-derived plasmid. Colonies carrying the cDNA in sense are recovered and the cDNA carrying expression cassette is subsequently excised and cloned into pCIB200 for use in plant transformations using *Agrobacterium*. For direct gene transfer, the cDNA-carrying expression cassette is transferred to the vector pCIB3064. Transformation to transgenic plants is undertaken using techniques well known in the art. For transformation of dicotyledonous species using binary *Agrobacterium* vectors such as pCIB200 see Alexander *et al.*, *Proc. Natl. Acad. Sci.* 90: 7327-7331 (1993), and for transformation of monocotyledonous species using direct gene transfer vectors such as pCIB3064 see Koziel *et al.*, *Biotechnology* 11: 194-200 (1993). Transgenic plants are screened for high-level expression of the appropriate cDNA by Northern or Western analysis. Plants which express high levels of the gene product are found to have enhanced resistance to plant pathogens.

Other promoters are suitable for the expression of these cDNAs in transgenic plants. These include (but are not restricted to) constitutive promoters (such as those from the ubiquitin and actin genes) and cell and tissue-specific promoters.

For genes involved in the signal transduction of SAR which may cause negative regulation of the SAR pathway, increased disease resistance can be achieved from the constitutive expression of cDNA in antisense to the gene coding sequence. The cloning and transfer of antisense sequences is undertaken in the same way as described above, except that the orientation of the cDNA is inverted to effect expression of antisense transcripts.

\*\*\*\*\*

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the spirit and scope of the present invention.

LIST OF REFERENCES

- Alexander *et al*, *Proc. Natl. Acad. Sci.* 90: 7327-7331 (1993)
- Alexander *et al.*, *Molecular Plant-Microbe Interactions* 5:513-515, (1992)
- Allen, G., *Plant Sci. Lett.* 26: 173 (1982)
- Altschul *et al*, *J. Mol. Biol.* 215: 403-410 (1990)
- Ausubel *et al*, in "Current Protocols in Molecular Biology, pub. by J. Wiley & Sons, New York (1987)
- Berk, A.J. *et al*, *Cell* 12, 721 (1977)
- Bevan, M.W. and Chilton, M-D., *Int. Rev. Genet.* 16: 357 (1982)
- Boulter *et al*, *Crop Protection* 9: 351-354 (1990)
- Brugidou *et al*, *Mol. Gen. Genet.* 238: 285-293 (1993)
- Carozzi *et al*, *Plant Mol. Biol.* 20: 539-548 (1992)
- Chester, K.S., *Quart. Rev. Biol.* 8: 275-324 (1933)
- Church and Gilbert, *Proc. Natl. Acad. Sci.* 81: 1991-1995 (1984)
- Claes *et al*, *Plant Cell* 2: 19-27 (1990)
- Davis, *et al*, *Proc. Natl. Acad. Sci, USA* 81: 2194-2198 (1984)
- De Pater and Schilperoort, *Plant Mol. Biol.* 18: 161-164 (1992)
- DeBlock, M. *et al*, *EMBO J.* 6: 2513 (1987)
- Deveraux *et al*, *Nucl. Acids Res.* 12: 387-395 (1984)
- Evans, D.A. *et al*, in: "Handbook of Plant Cell Culture", Vol. 1, MacMillan Publ. Co., 1983, p. 124
- Fischhoff *et al*, *Biotechnology* 5: 807-813 (1987)
- Greenberg *et al*, *Mol. Cell Biol.* 6: 1050-1057 (1986)
- Gubler, U. and Hoffman, B. J., *Gene* 25: 263 (1983)
- Hanahan, D., *J. Mol. Biol.* 166: 557-580 (1983)
- Hilder *et al*, *Nature* 330: 160-163 (1987)
- Klein, T.M. *et al*, *Nature* 327: 70 (1987)
- Kozziel *et al*, *Biotechnology* 11: 194-200 (1993)
- Kuc, J., *BioScience* 32: 854-860 (1982)
- Lagrimini *et al*, *Proc. Natl. Acad. Sci.* 84: 7542-7546 (1987)
- Lau and Nathans, *Proc. Natl. Acad. Sci.* 84: 1182-1186 (1987)
- Liang and Pardee, *Science* 257: 967-971 (1992)
- Margis-Pinheiro *et al*, *Plant Mol. Biol.* 17: 243-253 (1991)
- Mauch *et al*, *Plant Physiol.* 76: 607-611 (1984)
- Meins *et al*, *Mol. Gen. Genet.* 232: 460-469 (1992)

- 50 -

- Messing, J. and Vierra, J., *Gene* 19: 259-268 (1982)
- Mikkelsen *et al*, in "Advances in Chitin and Chitosan, ed. by Brine *et al*, pub. by Elsevier, Amsterdam (1992)
- Murashige and Skoog, 1962 *Physiol. Plant* 15:473-439
- Ow *et al*, 1986; *Science* 234: 856
- Paszowski, J. *et al*, *EMBO J.* 3: 2717 (1984)
- Potrykus, I. *et al*, *Mol. Gen. Genet.* 199: 169 (1985)
- Reich, T.J. *et al*, *Bio/Technology* 4: 1001 (1986)
- Reimann and Dudler, *Plant Physiol.* 101:1113-1114
- Rodriguez-Palenzuela *et al.*, *Gene.* 70:271-281, (1988)
- Ross, A.F., *Virology* 14: 329-339 (1961)
- Ross, A.F., *Virology* 14: 340-358 (1961)
- Rothstein, S.J. *et al*, *Gene* 53: 153-161 (1987)
- Saiki *et al*, *Science* 239:487-491 (1988)
- Schmidhauser and Helinski, *J. Bacteriol.* 164: 446-455 (1985)
- Simpson, R.J. *et al*, *Biochem. Intl.* 8: 787 (1984)
- St. John and Davis, *Cell* 16:443-452 (1979)
- Stahl, *et al*, *Nuc. Acids. Res.* 16: 3026-3038 (1988)
- Thompson *et al.*, *EMBO J.* 6: 2519-2523, (1987)
- Toki *et al*, *Plant Physiol.* 100: 1503-1507 (1992)
- Uknes *et al*, *Plant Cell* 4: 645-656 (1992)
- Uknes *et al*, *Plant Cell* 5: 159-169 (1993)
- Vaeck *et al*, *Nature* 328: 33-37 (1987)
- Van Loon, L.C. *et al*, *Neth. J. Plant. Path.* 88: 237-256 (1982)
- Ward, E. *et al*, *Plant Cell* 3: 1085-1094 (1991)
- Weeks *et al*, *Plant Physiol* 102:1977-1084, 1993
- Wen-jun and Forde, *Nucl. Acids Res.* 17: 8385-8386 (1989)
- White, R.F., *Virology* 99: 410-412 (1979)
- Williams *et al*, *Bio/Technology* 10: 540-543 (1992)

EP-A 223 452

U.S. Patent No. 4,751,081

WO 87/07299

EP-A 240 332

U.S. Patent No. 4,795,855

EP-A 249432

U.S. Patent No. 4,940,840

EP-A 392 225

**DEPOSIT**

NRRL B-21169

- 51 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Ciba-Geigy AG  
(B) STREET: Klybeckstr. 141  
(C) CITY: Basle  
(E) COUNTRY: Switzerland  
(F) POSTAL CODE (ZIP): 4002  
(G) TELEPHONE: +41 61 69 11 11  
(H) TELEFAX: +41 61 696 79 76  
(I) TELEX: 962 991

(ii) TITLE OF INVENTION: CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 29

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: Patent In Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1079 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACAAACCAAT AACACAAAC ATGGCTTTCA CAAAAATCTC CTTAGTCCTT CTTCTCTGCC	60
TCTTAGGTTT CTTTCTGAA ACTGTCAAGT CTCAAAACCTG CGGTTGCGCT CCAAACCTCT	120
GTTGCAGTCA GTTCGGTTAC TGTGGTACCG ACGATGCATA CTGCGGTGTT GGATGCCGAT	180
CAGGTCCTTG TAGAGGTAGT GGAACCCCGA COGAGGGGTC GGTCGGTAGC ATTGTGACAC	240
AAGGTTTCTT TAACAATATT ATCAACCAAG CTGGTAATGG TTGCGCGGGG AAAAGATTCT	300
ACACCCGTGA CTCTTTCGTT AACGCCGCTA ATACTTTCCC CAACTTTGCC AATTCTGTGA	360
CCAGACGTGA AATTGCTACC ATGTTTGCTC ATTTCACTCA CGAGACCGGA CATTTCTGCT	420
ACATAGAAGA GATTACGGA GCAACACGTA ACTACTGCCA GAGCAGCAAC ACACAATACC	480

- 52 -

CATGTGCACC GGGAAAAGGC TACTTCGGTC GTGGTCCGAT CCAACTATCA TGGA ACTACA	540
ACTACGGAGC GTGTGGTCAA AGTCTCGGTC TTGACCTTCT ACGCCAGCCC GAACTTGTGG	600
GTAGCAACCC AACTGTAGCT TTCAGGTCGG GTTTGTGGTT TTGGATGAAT AGCGTAAGGC	660
CGGTTCTGAA CCAAGGGTTT GGAGCCACCA TTAGAGCTAT TAATGGAATG GAATGTAACG	720
GTGGTAATTC CGGTGCAGTC AACGCAAGGA TTGGATACTA TAGAGACTAT TGTGGACAGC	780
TTGGTGTGGA CCCTGGTCCT AACCTTAGTT GCTAAAAAAC CTTTGAACCC AAACACGGAC	840
ATATGTGACG TGGCATGTAA TAAGTGAGAT ATACTAAAAT TTCACACGTA TGTACTTTAT	900
GTCGGGTCTC GGTGTTCCCT GCGTCACAAG CAAAAAATTG TTGTAATAAA CTTGTGAAAG	960
TGATTTTCTT TTCCTATGTG ACTTCTTATG AAAGAGAATT TTAAGATTGA TAGATGTTTG	1020
AGTTTGACAC TTGCAGTATC TGATTTTGGG ATACTCGCAT AAAAAAAAAA AAAAAAAAAA	1079

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 952 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAAACAACAT AACATGGCTT CCACCAAAT TTCCTTAGTC TTTTCTCTT GCCTCGTAGG	60
TCCTTGTATA GGCGCTGGAA CCACAAGCGC TGCCGAATCA GTCAGTAGCA TTGTGACACA	120
AGGTTTCTTT AACAACATTA TCAACCAAGC CGGTAATGGC TGCACGAGAA AAAGATTCAA	180
CACCCGCGAC TCTTTCATTG ACGCTGCTAA TAATTTTCCA AACTTTGCAA GTTCCGTTAC	240
AAGACGTGAA ATTGCAAGCA TGTTTGCTCA TGTCCTCAC GAGACGGAC ACTTCTGCTA	300
CATAGAAGAA ATAAAGAAAA GGTACGCTGG TAGGTGCGAC GAGAACGTAG AGCAGAGACC	360
ATGTCCATCA CAGAGTAAAG GTCCTCCGG TCGTGGTCAT CAGAGTCTCG GTCTCGACCT	420
TCTACGCCAG CCTGAGCTTG TGGGTAGCAA CCCAACTGTA GCTTTCAGGA AGGGTTTGTG	480
GTTTTGGATT AATAGCGTAA GGCCGGTGCT GAACCAAGGA TTTGGAGCCA CTATAAGAGC	540
CATCAATGGG ATGGAATGTA ACGGTGGGAA TTCAGGTGCG GTCAAGGCAA GGATTGGGTA	600
CTATAGAGAC TATTGTAGAC AGCTTGGTGT GGATCCTGGT CATAACCTTA GTTGCTAAAA	660

- 53 -

CGTTTTACAA ACGCATACAA CGTGACGTGT CACGTGATAA TGC GGAATAA GAAGTTACAT	720
TTCAAACAGG GCAAGGATTC AGAATCGCAA GTACATGAAT TAACATTCTG TAACTGTTAA	780
TTGTATCAAG TTGTGTTAAT ATATAAGAGC TAGGAGAATT GGTCTTTCTT ATGTCTAGCT	840
AGTAACCATC TATGTTTGTA GCTAGGAGCT ACTTGTTGTT TCTAGTTGTT TGTCTATCTA	900
AGCCAATCAT ACAAATCTA TGCTTATTCA CAAAAAAAAA AAAAAAAAAA AA	952

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1250 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAATTCGGCA CGAGCACACA CAATCCTGCC TATAGCCACC ATGTCTCCTC AGCCTACCTT	60
AGCCACCACC ACCACGCCTG CTACTGGGCC TAACCTTCC TATTACCAA CAGCTCCTGT	120
TAGCCAAGAG AGTATCCAGC GAACGGAGGT TCTTCTCCAC CTGTACGCCT ACCAGCACAC	180
CCAAGGAAAA CCAATGCTA ACCAGACTGT CATAGTGGAT CCAAGCTCC CCGCGTGTTT	240
CGGTGCCCTT GCTGCTAATG ACTGGACCAT ATATGATGGC ACTTGCACCA ATGCAAACCT	300
TGTTGCGCAC GCTCAAGGTT TGCACATTGG TGCTGGTATG ACCAAAGAAA ATTGGTTCAT	360
TTGTTTCAAC ATGGTGTGTTG TGGACCGAAG GTTTATGGGT TCCAGCTTCA AGGTGATGGG	420
AGATTTTCGA GGAAACGAAG GTGAATGGGC AATTGTTGGT GGGACGGGAG AGTTTGCATA	480
TGCACAAGGT GTCATAACCT TTAACAAGAC CTGGTCGGCC CAGGCAAATG TCAGGGAGCT	540
TCATGTTTCGT GCTTTGTGTC TGTCTTCTC AAAAGCACCG GAAACACCGT GCTCAAGGAC	600
ACCACGGGAG AGCTCTGTCA CCAAGATCGG CCCATGGGGT AAAATAAGTG GAGAATTTCT	660
TGACGTTCCC ACGACACCAC AACGTCTAGA GTGTGTGACC ATTGCGCATG GAGTTGTCAT	720
TGATTCACTT GCATTTTCCT TCGTCGACCA AGCTGGTGGG CAACATAACG TTGGCCCATG	780
GGGTGGGCCA TGCGGGGACA ACAAGGACAC GATTAACTT GTCCATCGG AGATTGTGAC	840
AGAAGTCTCT GGAACGATTG GTGTATTTGG AGCAGCCAAT GTCGAGTACA ATGCCATAAC	900
ATCACTAACC ATTACCACAA ATGTCCGCAC GTACGGGCCC TTTGGAGAAC CGCAGTGTAC	960

- 54 -

TCGTTTTCAGT GTTCCCGTGC AGGACAAAAG CAGCATCGTG GGTTCCTTCG TGTGCGCTAG	1020
GAAATACGTG GAGGCGCTCG GGGTTTACGT GTGTCCACCT ATTTCAAAGT AGTCCCGAAG	1080
TGCTTCACAC TCGGTGTGCC TTCCACCTTT CTATGTTGTG CCAATAAAGT AGGTTATATA	1140
CTTTTGGTGT AATGCTATGC CCCCCCCAA GTGTGATGGT TTTCATATCA GCAAGAAGTT	1200
TCTCTTGCTA CTATATGCAA AATAAAAAAA AAAAAAAA AAAACTCGAG	1250

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 340 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCTCGNGTG GCGAACGGAC GAGGAGTTCG CCGGGGAGAT GCTCGCCGCG CTCAACCCAC	60
ACATCATCAC CCGCCTGAAC GTGTTCCTC CGAGGAGCAC GNTTGAAGGG TACGGTGACC	120
AGACGAGCAA GATCACGGTG GAGCACATCC AACACAACCT CGGNAAGCTC AACGTCGACA	180
AGGCAATCGA CGCCAAGAGG CTCTTCATCC TAGACCACCA CGACAACCTC ATGCCTCACC	240
TGCTAAAGAT CAACAGTCTC CCAAACACAT TCCGTCTTAC GNCACCAGGA CGCTGCTCTT	300
CCTGCAAGAT GACGGGACTC TCAAGNCAAT CGNCATCGAG	340

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 436 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACATCGAGAA GGGATCATGA ATTGACCCGA TCCCCAACTC AAGAACCGGA ATGGGCCACC	60
AAAATCCCCT AATGCTACTC TACCCCAACA CATCTGACAT CGATGGTGAG AGCGCCACCG	120



- 55 -

GGATCACAGC CAAGGGCATC CCCAACAGCA TCTCCATCTG ATCGATGTTA TGCCGTTTTA	180
TGTTATTGTT GTATGTTTCC CATTGCAAAT AAGGAACGCT GCATGTGCAC GTTTCATGAG	240
TGGCCAGAAG CGTGCTCGTT CACGTTGAGG CGAGTTGTGT TTATTTTCGT GTTGATGAAG	300
TGTTTTGGCC GCAATGGAAA GCCCGTGGGA TGGCACGGAT GAAGGGTTGT GGTCGGCCAG	360
TGTGTTGAGC TGGACATATA TACGGAAAAA AATTGTATGA GACCGGGTCT CATAGACCCA	420
AAAAAAAAA AAAAAA	436

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1162 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AAGACACAAC CTTATTTTGC ATATAGTAGC AAGAGAACT TCTTGCTGAT ATGAAAACCA	60
TCACACTTGG GGGGGGGGCA TAGCATTACA CCAAAGTAT ATAACCTACT TTATTGGCAC	120
AACATAGAAA GGTGGAAGGC ACACCGAGTG TGAAGCACTT CGGGACTAGT TTGAAATAGG	180
TGGACACACG TAAACCCCGA GCGCCTCCAC GTATTCTAG CGCACACGAA GAAACCCACG	240
ATGCTGCTTT TGTCTGCAC GGGAACACTG AAACGAGTAC ACTGCGGTTT TCCAAAGGGC	300
CCGTACGTGC GGACATTTGT GGTAATGGTT AGTGATGTTA TGGCATTGTA CTCGACATTG	360
GCTGCTCCAA ATACACCAAT CGTTCCAGAG ACTTCTGTCA CAATCTCCGA TGGACCAAGT	420
TTAATCGTGT CCTTGTTGTC CCCGCATGGC CCACCCCATG GGTCAACAGC CCACAGCTTC	480
ACAAGCCAAT ACACACACCA ATATAGTCTC CTTTCCAGAG CTAGCTAGCA TCAATGGCTT	540
CTGGTGCTCA GATGAAGATG GTTGCCGTCG GCATGATGCT GGCCATCATG TTCATCGCTG	600
CAGCTCATGC AGAACCAGCG CCTGCAGAAA CTTGCATCGA CAAGACCGAA AAAGTTGGTC	660
TTGCCACTGA CTGCATCTGC TCCAAGAACT GTGCTTGTC AGGAAAGTGC ATCTTAGAAG	720
GCGGCGAGGG TGGCGAAATC CAGAAGTGCT TTGTGGAATG CGTGCTGAAA AATGACTGCA	780
ACTGCAATGC TAAGCACCAC AGTGCCGAG CCCCTCAGTA AGGAGACTCC TGATTGGAGC	840
TTCTTCAGCC ATGCTATGAG GTCTGCGTCC ACAAGTCCAC AGTCAACATT TGAGTAAATT	900

- 56 -

AATAAACCTC TTATACCTGC GTTCTTGCCA TGCCTTATT GCTTGACGCG AATGTATGTT	960
GAGAGTTGGT TTCCCGGTCA CAGCTCATAT ATATGCTGCC AGTCAAGTGT ACGACGACTG	1020
CCACATGTAT TGCTGGAAAT GAATATGTAT GTGTATTGT CCTTTTCGTC ATTTGGGTCA	1080
TTTTAAAAAT AAGTTGTATG CTGGAGCATG ACAACAAGTG TATTTGGCTA CTATAATATT	1140
ATTAATGATA TGATGTCTTG TT	1162

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1371 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATCCACCTCG ATCCAGCCAT GCTGTCTCC AAGTCTTCTT TAGCTGTTGC TCTAATCCTT	60
GTGGTCACCC TGACAGATGT GCTACCGCTG GTATCTTCTT CACGTGGCCT CGTTGGGGTA	120
CCCACTGATG GGGCACTGGA GGAATCTCTG TTGATGATGG AGAGATTCCA TGGCTGGATG	180
GCAAAGCATG GCAAGTCGTA TGCGGGAGTC GAGGAGAAAC TGCGGCGGTT TGACATATTC	240
CGCAGGAACG TAGAGTTCAT CGAGGCGGCG AACCGAGATG GCAGGCTCTC GTACACCCTC	300
GGGGTGAACC AGTTCGCGCA CCTCACCAC GAGGAGTTCC TTGCCACGCA CACCAGCCGC	360
CGTGTGGTGC CGTCAGAGGA GATGGTGATT ACAACTCGCG CTGGCGTTGT TGTCGAGGGT	420
GCCAATTGTC AGCCGGCGCC AAATGCTGTG CCTCGTAGCA TCAATTGGGT GAATCAAAGC	480
AAAGTCACCC CAGTCAAAA TCAAGGAAA GTATGCGGGG CTGCTGGGC TTTTCTGCC	540
GTGGCCACGA TCGAAAGCGC CTACGCGGTC GCCAAGCGAG GCGAGCCGCC GGTCTGTCC	600
GAGCAGGAGC TCATCGACTG TGACACAATC GACAGAGGCT GCACGAGCGG CGAGATGTAC	660
AATGCCTACT TCTGGGTCTT GAGGAACGGC GGCATCGCCA ACAGCTCAAC GTACCCCTAC	720
AAAGAGACTG ACGGCAAGTG CGAGAGAGGG AAAGTGCAGG AACACGCGGC CACGATCAGG	780
GACTACAAAT TCGTCAAACC CAACTGCGAG GAGAAGCTCA TGGCAGCCGT GCGGTGCGA	840
CCCGTCGCCG TCGGGTTCGA CTCCAACGAC GAATGCTTCA AGTTCTACCA AGCTGGTTTG	900

- 57 -

TACGACGGCA	TGTGCATCAT	GCACGGGGAA	TACTTTGGCC	CGTGCTCGTC	CAACGACCGC	960
ATCCACTCCT	TGGCCATTGT	CGGGTACGCC	GGCAAGGGGG	GCGACAGGGT	CAAGTACTGG	1020
ATCGCCAAGA	ACTCGTGGGG	CGAGAAGTGG	GGAAAGAAGG	GCTACGTCTG	GCTGAAGAAG	1080
GATGTTGATG	AGCCGGAAGG	CCTCTGCGGC	CTTGCAATTC	AGCCGGTATA	TCCTATAGTC	1140
TGATCTGATC	TGACGAGATC	GACTGCACTG	GGCGTGCATG	AAACCTACGG	AAATGGCATT	1200
CACCTATATT	TTGGGTTGCT	CTGTATGCAT	GGATGCGCCT	ACTATATTTT	ACTACATATA	1260
TATTCATCTC	CCGCTAATAA	AACTACATGT	CCTTGTATCC	ATTTATGCAC	GTTTATCCAT	1320
ATCTTGAATA	AATTGGATGG	ATTGGTTATC	CAAAAAAAAA	AAAAAAAAAA	A	1371

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 723 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAACCGGGAA	CCGGAGGCCT	CCGACCCCTC	ACCTCGCTGT	GGTTGACAAC	ATCGACCTGT	60
GCCCAAGACC	TCGGGGAACG	CTACCAACTC	CGAACCCAAG	TATGTCCCGG	CCTTCGACGA	120
GGCCGACGTC	AAAGAAGGAG	GTGAAGGGCG	TTCTGTACCC	ACGAGGCGAC	GCACGTGTGT	180
CAGTGGAACG	GGCAGGCCAG	GTCAAACGGC	GGGCTCATCG	AGGGGATCGC	CGACTACGTG	240
CGGCTCAAGG	CCGACCTCGC	GCCGACGCAC	TGGGCCCCGC	AGGGGAGCGG	CGACCGTTGG	300
GACGAGGGGT	ACGACGTGAC	GGCCAAGTTC	CTGGACTACT	GCGACTCCCT	CAAGGCCGGG	360
TTCGTGTCCG	AGATGAACAG	CAAGCTCAAG	GACGGATACA	GCGACGACTA	CTTCGTGCAG	420
ATCCTGGGGA	AGAGCGTGGA	CCAGCTGTGG	AACGACTACA	AGGCCAAGTA	CCCCCAGCC	480
CCAGGGCTGA	TCGACGATGC	ATGCAGTTTG	TTGTTGTATG	TGTACCGGTC	TTCGTCTACA	540
TACAGATACA	TTATAGTACT	TGTATTACTG	TACAATTTAT	GTA CTGCCTG	GAATGGAATA	600
AATCAGCGTT	GGCACGGTGT	GTGTTAACGA	ATTGACGAGA	CAAAGGGACC	GTCTATAGGT	660
CATGTCATCG	GTTGCCTGAA	ATACATTGAA	CATCATCACT	TTCTTTACAG	CAAAAAAAAA	720
AAA						723

- 58 -

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 765 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```

AAATGACAAT TGAAATTTAT TAAAGTAATA TGATCTAATA TGTTCAAACA AGCTCAATCA      60
CAATTTAATT TAATTAATAT TGACCGCGCA TCGCGCGAGT ACGACTACTA GTTCAATTTA     120
GAAAGCTGGA GCCATTGCCG GAGTAATCAT GAAATGGCG GAGTTGACTT CCGATTCCGG      180
TGATGGTGCG AGTATTCCCG AGCTTTTGAT CTCGCCATT CCACGGCGTG CATTTCACC      240
TTGTTCTTCA CACAATCTGG GAAGGTATAC ACCTTCTCCA GCAGCAAGGT TGAAGTAGAG     300
ATCAATAATA TTAGGGCAAT GTTTGTAGCA CTGAGGGGAG CACAGCTTCT GGGTAAAGTG     360
GCACTCGAGG AGAGAATCAG AAGAATTCOG AAAGTATTC TATCAACACC GCAAGCCTGA     420
ATACACTGGT CGGTTTCAAT CCAGTCTTTG AGCTTATCAG CCTCAATTTC CGACGTCTTA     480
CATGTATATA CTCTTTCACC ACTCCTTTGG AGACGTTTCT CTAACACGCA ACGCTTCCCA     540
GTACTTGAAA TCGCAAAAGC GCATGAGTCC TTGTTTAGAT TCTCGCATGT TATGCTCCCT     600
AGAGTGACTT GAACACAGAA GACAAGTGCA CAAGCAACAA TAGCCAAAGT AAAGTTGTGG     660
AATGAAGCAA CCATGGTGAA AAATCTAGCA ACAATTGATC AGACTATAAA TTTTTTCTGA     720
GTATATATAG CTCCTTTTGG CTTTCGACTC TCCTTTTTTG TGGTT                      765

```

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1054 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

- 59 -

GAATTCGGCA CGAGTTTGCG TATACATCAA TACTCCGGCT AAATCGGCAA ATATATCCAA	60
CAAATTAAGC CTTTTAAACC AATCAGTACG TTATCAATAT CATCAATGTC AGATCGTATT	120
GATGTCCTGG CTATGATGAT ATCAGATGAG GAATTTGCAG AGAAACTGCA GATGCAACAT	180
GTTCTCGGAG CCTCTTATGA AAGTTCCCAA GGATTCTGTG GAATTTGCAA TAAGAAAAAG	240
GGAGCAGATC GAATGTTCAA ACTCCAAATC TGCTCTCGTC ATTCTTTCTG TTCTCACTGC	300
ATAGGTCTTT ATGCCCAATC CAAGATTGCG GACAACGTTT TCCCTATAAC TTGTCCAGGT	360
TTGAGATGTC GTGTCGTAAT TCAACCCGAA TCTGTAGGT CTATCATTCC AGTAAATACA	420
TTTGCAAGGT GGAAGAGGG CTCGGCCGAG TCAGCTATTC CTAATGGTGA AAAATTCTAT	480
TGTCCTTATA AAAGATGTTT AGGGCTGTTT ATTCATGATC GACATGAAGA GATCGTTCAG	540
TGCATTTGTC CTCTGTGCAA AAAACTGTTT TGTGCGAAGT GCGAGTCCC TTGGCACACT	600
GGGCGTGATT GTGACAAGTT TCAAAAGGTA GAAAAAGACA GAGAAGATGA GCTAAAACCT	660
AAGCTGCTTG CTGAGATCAA AAAATGGAAG GAATGCCCTA AGTGCAAATC CATTGTGGAG	720
AAGGTTGATG GCTGTATACA CATGACTTGC AGGTGTAAAA TGAATTTTGT CTACGTATGT	780
GGAGGAACGT GGAGCGAAG GCATTGGAGT TGCCAAGACT AGTTATGATA TACTATCGGA	840
TTGCCTCTTC ATACCAATCG GATGCTGAAT ATTTGTGTGT CAGAATAAAA ATTATGTTGT	900
TTAGTTGGTT GTATTATGTC TTTAATTGGT TGTCCATGGT TTGTATTAGG AGCATTATAT	960
ATAGTACGAG TTGGTCCTTG CATAACATTT ACTTGATGTT GATTATAATA TGCTCGAGTT	1020
GGAGACCCCA AAAAAAAAAA AAAAAAACG CGAG	1054

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 760 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCGTGCCGC TTTAAGTGTG CTCGAACCTC CTA CTGGTAA TGAGGATGAT GATGACCTGG	60
AATTTGAAAA TGTCCACTGG AATGGTTCAG ATATGGCATC CGATGATACT CAAAAATCTC	120

- 60 -

ATAGACCAAG GCACCGCGTA CATAAATCGT CTGGTTCCCA CAAGGCCATG AGCCGCTCCC	180
TTTCATGTGA CTCGCAATCA AAAGGATCTA TTTCTACACC TCGTGGGTCC ATGGTTGACC	240
TAAGCAAACCT CGAGATGGCT GCACTGTGGA GATATTGGCG ACACTTTAACT CTTAGGGAAG	300
CTATACCTAA CCCCTCGAAA GAGCAACTAA TTGATGTTGT CCAGAAGCAT TTCACATCTC	360
AGCAATTGGA CGAGTTGCAG GTAATTGTGG GATTTGTTCA AGCTGCCAAG AGACTCAAGA	420
CAGTCTGCAA ATGACTCAAG GGAACGAACC CCTTTTGGGA ACGCCCCTAA CACTAACAAA	480
TAGTGGTACC TTTGTGTCCA CTTGCTATTG CGCAGTAATA TGTGTAGTAT AATATGTATA	540
CTCCTGTTTA TCCCTTATGT CTTGCTTAGA GTTGTAGTTT ATGTAGATAA ATGAACTGCT	600
GAACTAACTC TGGAGGTCTC CTTCTGGTTG AATGTACTTC CTCGCGAGTG GGGGAAGTAC	660
TAGTACTTAA TCACATGTAT AGTCTAATGT CTTAAACTGT TAAATCATCA TTCAACCAAC	720
TGAATCTTGG TTTGGTATTA GTGACCCTCG TGCCGAATTC	760

## (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 396 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGCCGCTTTA AGTGTGCTCG AACCTCCTAC TGGTAATGAG GATGATGATG ACCTGGAATT	60
TGAAAATGTC CACTGGAATG GTTCAGATAT GGCATCCGAT GATACTCAA AATCTCATAG	120
ACCAAGGCAC CGCGTACATA AATCGTCTGG TTCCCACAAG GNCATGAGCC GCTCCCTTTC	180
ATGTGACTCG CAATCAAAAG GATCTATTTT TACACCTCGT GGGTCCATGG TTGACCTAAG	240
CAAACCTCGAG ATGSCITGCAC TGTGGAGATA TTGGCGACAC TTAAACCTTA GGGAGGTATT	300
CCTAACCCCTC GAAAGAGCAC TTATTGATGT GGTGAGAGCA TTCANATCTC AGAAATGGGC	360
GAGTGGAGGT ATTGTGGATT GGTCAAGCTG CAAGAG	396

## (2) INFORMATION FOR SEQ ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 653 base pairs
- (B) TYPE: nucleic acid

- 61 -

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTTGTTTGGT TGTTTGAGTT ATTTTGCTTC TAAGAACTTT GTGAGAAATG GCTGCTAACG	60
ATGCTACTTC ATCCGAGGAG GGACAAGTGT TCGGCTGCCA CAAGGTTGAG GAATGGAACG	120
AGTACTTCAA GAAAGGCGTT GAGACTAAGA AACTGGTGGT GGTCGATTTT ACTGCTTCAT	180
GGTGCGGSCC TTGCCGTTTT ATTGCCCAA TTCTTGCTGA CATTGCTAAG AAGATGCCCC	240
ATGTTATATT CCTCAAGGTT GATGTTGATG AACTGAAGAC TGTTTCAGCG GGAATGGAGT	300
GTGGAGGCAA TGCCAACCTTT TGTCTTCATT AAAGATGGAA AAGAAGTGA CAGAGTTGTT	360
GGTGCCAAGA AAGAGGAGTT GCAGCAGACC ATAGTGAAGC ATGCTGCTCC TGCTACTGTC	420
ACTGCTTGAA TCTCCTTAAT CAAGGGGATG ATATCCCAT ATTAGTAGTA TTGCTTTTTG	480
TAATAACCAA GTAACCTGTT CGAATTCAC ACTATGGATC ACTGTATGGT TGTACTATCC	540
ACCATGTTTT TATTGCTTTT GTGAACCTTG TCTTGTGCT TGGAACTGA TTTGTGCATT	600
ACTGGTGTA GGCTATATGC CCAATTCYAC AAAAAGACTA CTTTTAGATT TCT	653

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1697 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTCAAAATT ATTGGATCCT AGTTTAAAGT GGAACCTCTA ATTAATCAAT CATTTTCATGA	60
TCAACCTTTA CTAACATCAC CATTTTCCTT GACAACATTA TTTTCCGACT TACCATTTTC	120
CTTAACACCA TTACCCTCTG GATTTTCCTT GACAATATTA CTCTCCGACT TCTCATTTTC	180
GTTCACTTTA TCAGTAATCT TGGCTTCTTC AGCAGCTGCT TCTTTCTCTT TCTCTTTCTC	240
TTTCTCAGCC CTTTCTCTTT CTCTTCCTC AGCCTTCAAT TTTGGCTCCT CCTTTGCAGT	300

- 62 -

TTCAAGAGCT TTAATCAGTC TCTCCAAACA AGTGTGTTGCA TTTTCCTTAG AAGACTTGGG	360
CATTAAATTC TCAGCAACAT CAGCAGGAGT AATATTAGTT TCCCCAATA AATGACGAAT	420
CTCAGGAAAA TGATCATGAG ACTCAATATC TAGATAATTA TTTGCAAGCA CCTTGAAGGA	480
GTCAAAGCAA CAGTATGATA ACACAATGTG TTTATCCATC CTCCCCCTCC GAATTAAAGC	540
AGGGTCAAGC TTTTCCACAA AGTTGGTAGT GAAACAATA AGCCTTTCAC CACCAATAGC	600
TGACCATAAC CCATCAATAA AGTTCAAAAG CCCAGATAAA GTCACCTCGC TTTGCTTTTT	660
CTCCTCTCGA TTTTTCATCT TCTCCTTGAC GGCATCTTTC TCGTCTTTTA CTTCCTCTTC	720
CTTGTCGTCT TTCTTCTTCT CCCTTTGGCC GGTAAAGTCA AGCGAACAGT CGATGTCTTC	780
AATCACAATG ATAGACTTAC TAGTAGTATC TATTAATAAC TTTCTTAGCT CGGTGTTGTC	840
CTTAACCGCT GTCAATTCAA GATCATAGAC ATCATATTGT AAGAAGTTAG CCATTGCAGC	900
AATCATGCTA GACTTACCGG TTCCTGGAGG ACCATATAGA AGATAACCAC GCTTCCATGC	960
CTTGCCAATC TTGGCATAAT AGTCTTTTGA CTGCTAAAT GTTTGAAGGT CATCCATAAT	1020
CTCTTGTTTC TTGTTTGGCT CCATGGCTAA AGTATCAAAT GTTGATGGAT GTTCAAACAC	1080
TACTTGGCTC CACATTCTCC TCCTGTATCC ATACCCACCA TCTCCCTTAC TGTTTGTGTA	1140
CAACTTTCTC TGCTTTTCTC TTAAGTAAAT TGCCTTCCCT TCGTCCAATA CATACTTCAA	1200
GTATGAAGCG GTGATAAGCT CGCGGTTCTT TCTGTGAAAC TTGAGTTTGA AATACCTCTT	1260
CTCATCCTCC CTAGGGTACC AAGAAATTGT CTGTCTGCTG GCTACTTGTT GGCTAGAAAT	1320
CCACCAGACT TTCTCGCCTT TATATTCATC GGTTACCTCC TCATGATCAT CCATGGTTAG	1380
TACAAGAGAT TGGCCATCTT TCACTACATT GGCTTTGAGA CGCTTAGCTT GTGTGGAGGA	1440
GTTCTTGCTT AGGTACCTTT CAATTGCTAC ATAAGCTTTG CTACGCTCGA ACCAGCCATC	1500
AGTTTCATAC TCATGAAAAA TAATGTGCAT ATAAGGGTAG AAATAGCTCA CGAGTTTATC	1560
GGTATACCTC CTAATATGAC CACGAAGTTC GTGAGGAAAA TAGTTCTGGT ACATGGTCCA	1620
GGCAAACATG ATTGTTGCAA TAGCTGGACC CAACTGAGTC CAAACATCTT GCATCATCAT	1680
CATCATCTAA TTTCTCT	1697

## (2) INFORMATION FOR SEQ ID NO: 15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 654 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



- 63 -

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTAACCTTGA GATAAGCATT AAAAAAATC AATGGCAGGG AAGGTTGAGA AAGTGCTTGC	60
AGTACTGATG CTTGCAATGC TTCTGTTTTC GGAGCATTTT ATGGCTGCTA ATCATGAAAT	120
TAAACAACCT GAAGATAACT CTATTAGCCC TTTCTGCTTA ATAAAATGTT TATATGGATG	180
CAGGGGGTTG CCACCTGCAA AAGCAGCCAT TTGTGCAGCT CAATGTTTGT TTAAGTGCGC	240
TGTCCAAGAT GAGGCCAATA TAGCTGAAAC TAAGGGCATA ATAGGTGAGA CTGCATACAA	300
CCAGTATGAT GTTGGATGTG CCGTTGGCTA CTGCTCTGAG TTCCTGTTGA ATTATGATGA	360
GAGGAGGTTC AACTGCTGCA TGAATACTG TCGCGAGGGC AAAATGACCT GTCCTGTTGA	420
GGCTGCACCT TGAAGAAATG GTTGCCCTAA AATTATCGCC TCATCAAATG GAAGTACACT	480
GCTTTTTTCTA CTTCGGTGT TTAGTAGTAG TAGTAAATAA GTGAGGCATG TTACGTACTC	540
TTATGTTTTG TAATAATTAT GCTTTTAAAT AATGTAATCT GTCTGTGTGC ATACAATGCA	600
CACGACGCTA GCTACTACTT TTTATCTACT AAAACGAAA AGTAACTTAT TTCT	654

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1031 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAGACAAATA CCTATAATAA GCCATTCATA ATCTTCITGC TTCTTGTTC GAATAATGGG	60
GAATCTTTTC TGTGCGTGC TTGTGAAGCA ATCAGATGTT GCGGTCAAGG AGAGATTTGG	120
CAAATTCCAA AAAGTACTTA ATCCAGGTCT CCAATTTGTT CCATGGGTCA TCGGTGATTA	180
CGTCGCCGGT AACTGACCC TTCGTCTTCA GCAACTCGAT GTTCAGTGTG AAACCAAAAC	240
AAAGGACAAT GTGTTGTGA CAGTGGTTGC ATCCATACAA TACAGAGTCT TAGCTGACAA	300
GGCAAGTGAT GCTTTTTTACA GACTCAGCAA TCCAACCACC CAAATCAAAG CCTACGTCTT	360

- 64 -

TGATGTGATC AGAGCATGTG TTCCAAAGCT GAACTTGGAC GATGTGTTTCG AGCAGAAGAA	420
TGAAATTGCC AAATCTGTGG AAGAAGAGCT AGACAAAGCC ATGACTGCTT ATGGTTACGA	480
AATCCTTCAA ACCCTAATTA TCGACATTGA GCTGATCAA CAGGTTAAAC GTGCCATGAA	540
CGAAATCAAC GCCGCGGCGA GGATGAGAGT GGCAGCGAGC GAAAAAGCAG AGGCTGAGAA	600
AATCATTCAG ATCAAAAGAG CAGAGGGTGA AGCAGAGTCA AAGTACCTGT CGGGACTCGG	660
AATCGCTCGG CAGAGACAAG CGATCGTGGA CGTCTTGAG AGACAGTGTT CTGGGGTTCG	720
CAGGAAACGT GCCAGGGACG TCAGCGAAGG ATGTGTTGGA CATGGTGATG ATGACTCAGT	780
ACTTTGACAC AATGAGAGAT ATCGGAGCAA CTTCTAAATC CTCTGCGGTG TTTATCCCTC	840
ACGGTCCAGG CGCCGTCTCT GACGTGGCAG CGCAGATTCG AAATGGATTA TTACAGGCCA	900
ACAATGCCTC CTAATCACTC AAGTCAAATT GTCTTGGTCG TCTCTTTATA TATTTTCGTA	960
TCTTCTTATT AAAAAGGTAA ATTTGACTTT TAATATAATG GTGTGCTTAT TCGGAAAAAA	1020
AAAAAAAAAA A	1031

## (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 860 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGGAACAAAA GCTGGAGCTC CACCGCGGTG GCGCCGCTCT AGAACTAGTG GATCCCCCGG	60
GCTGCAGGAA TTCGGCACGA GCAACTTAGA AAAATGAATT TTACTGGCTA TTCTCGATTT	120
TTAATCGTCT TTGTAGCTCT TGTAGGTGCT CTTGTTCTTC CCTCGAAAGC TCAAGATAGC	180
CCACAAGATT ATCTAAGGGT TCACAACCAG GCACGAGGAG CGGTAGGCGT AGGTCCCATG	240
CAGTGGGACG AGAGGGTTGC AGCCTATGCT CGGAGCTACG CAGAACAAC T AAGAGGCAAC	300
TGCAGACTCA TACACTCTGG TGGGCCTTAC GGGGAAACT TAGCCTGGGG TAGCGGTGAC	360
TTGTCTGGCG TCTCCGCCGT GAACATGTGG GTTAGCGAGA AGGCTAACTA CAACTACGCT	420
GCGAACACGT GCAATGGAGT TTGTGGTCAC TACACTCAAG TTGTTTGGAG AAAGTCAGTG	480
AGACTCGGAT GTGCCAAAGT GAGGTGTAAC AATGGTGGAA CCATAATCAG TTGCAACTAT	540

- 65 -

GATCCTCGTG GGAATTATGT GAACGAGAAG CCATACTAAT GAAGTAATGA TGTGATCATG	600
CATACACACG TACATAAAGG ACGTGTATAT GTATCAGTAT TTCAATAAGG AGCATCATAT	660
GCAGGAYGTA TCAATATTTA TCAAATAATA CAAATAAGAG CTGAGATTAC GAGAATCTAT	720
TTAAATTAAA AGTTACATAC TTAATTATTA TAGTTATATA TGTAATAATAT GTGGCCTTTT	780
TAAAGTTAC ATAATTAATT ATTATAGTTA ATGTCTTTCA AAAAAAAAAA AAAAAAACT	840
CGAGGGGGGG CCCGGTACCC	860

## (2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTTGGCGTCT TCCATTTTTC TAAGTTGATA ATGG

## (2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TATCAACTTA GAAAAATGGA AGACGCCAAA

## (2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1064 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- 66 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAACAATGAA CATTAAGGTA TCATTACTTT TCATTCTACC AATATTCTTG CTTCTCCTAA	60
CCAGCAAAGT AAAAGCTGGA GATATTGTAG TCTATTGGGG CCAAGATGTA GGAGAAGGTA	120
AATTGATTGA CACATGCAAC TCTGGTCTCT ACAATATTGT CAACATTGCC TTTTATCTT	180
CTTTTGGCAA TTTCCAAACT CCTAAACTTA ACTTAGCTGG CCATTGTGAA CCATCTCTG	240
GTGGTTGCCA ACAGTTGACA AAAAGCATCA GACATTGTCA AAGCATAGGC ATTAATCA	300
TGCTCTCCAT TGGAGGTGGA ACTCCTACCT ACACATTATC CTCAGTTGAT GATGCCAGAC	360
AAGTTGCTGA TTACCTGTGG AACAAATTTTC TCGGCGGCCA ATCATCTTTT AGGCCACTTG	420
GAGATGCTGT ATTAGATGGC ATAGATTTTG ATATTGAACT TGGCCAACCA CATTATATTG	480
CACTTGCCAG GAGACTTTCA GAACATGGCC AACAAAGGTAA AAAATTATAC TTAAGTGCAG	540
CACCACAATG TCCTTTTCTT GATAAACTTC TTAATGGTGC ATTGCAAACT GGTATTATTG	600
ACTATGTTTG GGTCCAATTT TACAACAATC CCGAGTGCGA GTTCATGAGC AATTCAGAAA	660
ATTTCAAGAG GAGGTGGAAT CAGTGGACAT CAATCCCTGC AAAGAAGTTG TATATTGGAC	720
TTCCAGCAGC CAAGACAGCC GCGGGTAATG GCTATATTCC AAAGCAAGTG CTAATGTCAC	780
AAGTTTTACC ATTTCTAAAG GGGTCTTCAA AATATGGAGG TGTCATGCTT TGGAATAGAA	840
AATTTGATGT CCAATGTGGC TATAGCTCTG CTATCAGGGG TGCTGTTTAA GTTCTGAATG	900
AACAAGGCGC CCCTGAATCG CTATAAGCCA TCGTTAAGGC CTAAATAAAG CAAGTTAATT	960
TGCTGTTATC TGCCTAGAAA GTACTTAAGT TTTAATTTGT ACTGATGAAA ATGTGAAGGT	1020
CATCTTGTTT CCTTCTTGAT AATAGTAGTA CTATGGTTCT CTTT	1064

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1018 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

- 67 -

TGAAGTGTTA GCACACACAC ACAAAAAAAAA CTTAAAGTTA TGATCAAATA TAGTTTTCTT	60
TTGACAGCAT TAGTGCTATT TCTTCGAGCA TTAAAACTAG AAGCAGGGGA TATAGTAATA	120
TATTGGGGCC AAAATGGGAA TGAAGGTAGC TTAGCTGACA CTTGTGCAAC AAATAACTAT	180
GCCATTGTCA ATATTGCTTT CTTGTAGTT TTTGGGAATG GCCAAAATCC AGTGCTAAAT	240
TTAGCTGGTC ATTGTGATCC AAATGCTGGT GCATGCACTG GCTTAAGCAA TGACATTAGA	300
GCTTGTCAAA ACCAAGGCAT CAAAGTTATG CTTTCTCTTG GTGGTGGTGC TGAAGCTAT	360
TTTCTTTCTT CTGCTGATGA TGCTAGGAAT GTGGCAAAT TTTGTGGAA CAATTATCTT	420
GGAGGTCAAT CAAACACACG TCCACTAGGA GATGCAGTTC TAGATGGAAT TGATTTTGAT	480
ATAGAAGGCG GGACAACACA ACATTGGGAT GAATTAGCAA AAACCTCTATC ACAATTTAGC	540
CAACAAAGGA AAGTATACTT AACTGCAGCT CCACAATGTC CATTCCCAGA TACATGGTTA	600
AATGGGGCAC TTTCCACTGG CTTATTTGAT TATGTTTGGG TTCAATTTTA CAATAATCCA	660
CCGTGTCAAT ACTCCGGTGG GAGCGCGGAC AATTTAAAAA ATTACTGGAA TCAGTGGAAC	720
GCGATTCAAG CTGGAAAAAT TTTTCTGGGA TTGCCAGCAG CTCAAGGAGC AGCTGGAAGT	780
GGTTTTATAC CATCTGATGT TCTTGTTTCT CAGGTTTAC CATTAAATTAA TGGTTCACCA	840
AAGTATGGGG GTGTTATGCT TTGGTCTAAA TTTTATGACA ATGGTTATAG CTCTGCTATT	900
AAGGCTAATG TTTGAGATAT ATGATCATAG CTAGTCAGCT TGTATTAATA TGATGACGTC	960
AATAATGTTA TATTATAAAC TATATAGTAC TCAATAATAA GGCTTTGAAA GTTACTTA	1018

## (2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGGATCCCTG CA

12

## (2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs

- 68 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ATAGTCTTGT TGAGAGTT

18

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TCACGGGTTG GGGTTTCTAC

20

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGGAGATGGT TTGGTGGA

18

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 69 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ATACGTTCTA CTATCATAGT

51

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 7 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

His	Phe	Cys	Tyr	Ile	Glu	Glu
1				5		

(2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Ile	Arg	Ala	Ile	Asn	Gly
1				5	

(2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 731 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 40..528

- 70 -

(D) OTHER INFORMATION:/note= "PR-lmz cDNA clone"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```

GAATTCCGGG AACTAGTAAT TATCAGCAAA CAACAAACAA TGGCACCGAG GCTAGCGTGC      60
CTCCTAGCTC TGGCCATGGC AGCCATCGTC GTGGCGCCGT GCACGGCCCA GAACTCGCCG      120
CAGGACTACG TGGACCCGCA CAACGCGGCG CGCGCCGACG TGGGCGTCGG GCCGGTGTCC      180
TGGGACGACA CCGTGGCCGC GTACGCGCAG AGCTACGCGG CGCAGCGCCA GGGCGACTGC      240
AAGCTGATCC ACTCCGGCGG GCCCTACGGC GAGAACCTCT TCTGGGGCTC CGCCGGCGCC      300
GACTGGTCGG CGTCCGACGC CGTGGGCTCC TGGGTGTCCG AGAAGCAGTA CTACGACCAC      360
GACACCAACA GCTGCGCGGA GGGGCAGGTG TGCGGCCACT ACACGCAGGT GGTGTGGGCG      420
GACTCCACCG CCATCGGCTG TGCCCGCGTC GTCTGCGACA ACAACGCCGG CGTCTTCATC      480
ATCTGCAGCT ACAACCGGCC GGGCAACGTC GTCGGCGAGA GCCCCTACTA GACTGTCATG      540
CATACTACAA TTATATATAT ATACGCTTAA TATTAAC TTC ATGTCATG CATATTATAA      600
ATAGTGTGTG CAACTCTGTA TATCATTATA TTACGATGAT TATATTGTTG ATGAACATAA      660
TAAGTCAATA AAACCATGCA GGCGATTTGT GAGCCAAAAA AAAAAAAAAA AAAAAAAAAA      720
AAAAACTCGA G

```

731

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 163 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```

Met Ala Pro Arg Leu Ala Cys Leu Leu Ala Leu Ala Met Ala Ala Ile
 1             5             10             15
Val Val Ala Pro Cys Thr Ala Gln Asn Ser Pro Gln Asp Tyr Val Asp
      20             25             30
Pro His Asn Ala Ala Arg Ala Asp Val Gly Val Gly Pro Val Ser Trp
      35             40             45
Asp Asp Thr Val Ala Ala Tyr Ala Gln Ser Tyr Ala Ala Gln Arg Gln
      50             55             60
Gly Asp Cys Lys Leu Ile His Ser Gly Gly Pro Tyr Gly Glu Asn Leu

```



- 71 -

65		70		75		80
Phe Trp Gly Ser Ala Gly Ala Asp Trp Ser Ala Ser Asp Ala Val Gly						
	85		90		95	
Ser Trp Val Ser Glu Lys Gln Tyr Tyr Asp His Asp Thr Asn Ser Cys						
	100		105		110	
Ala Glu Gly Gln Val Cys Gly His Tyr Thr Gln Val Val Trp Arg Asp						
	115		120		125	
Ser Thr Ala Ile Gly Cys Ala Arg Val Val Cys Asp Asn Asn Ala Gly						
	130		135		140	
Val Phe Ile Ile Cys Ser Tyr Asn Pro Pro Gly Asn Val Val Gly Glu						
	145		150		155	160
Ser Pro Tyr						

## (2) INFORMATION FOR SEQ ID NO: 31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 831 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 72..587
- (D) OTHER INFORMATION: /note= "PR-5mz cDNA clone"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GAATTCCGGA GCCTAGCTCT ATAGCTCGAG TATTGCTTGC TCTCCACAAG AAACAAGGCA	60
GAGCACCAAC AATGGCGGCC GCGTCCTCGG TCCTCCTGCT GCTCCTGGCC GCCGCCTTGG	120
CTGGCATGAG CGCCAAAGCC GCCACCTTCA CCATCACCAA CAACTGCGGG TTCACCGTGT	180
GGCCCGCGGC CACCCCGGTG GCGGGGGGCA CGCAGCTGAA CCCGGGCGGG ACGTGGACCG	240
TCAACGTGCC GGCCGGCACC AGCTCCGGCC GCGTGTGGGG CCGCACCGGC TGCTCCTTCA	300
ACGGCAACAG CGGGAGCTGC CAGACGGGCG ACTGCGGCGG CGCGCTGGCC TGACACGTGT	360
CGGGGCAGCC GCCGCTGACG CTGGCCGAGT TCACCATCGG CGGCAGCCAG GACTTCTACG	420

- 72 -

```

ACATCTCGGT CATCGACGGC TACAACCTCG CCATGGCCTT CTCCTGCAGC ACCGGCGTGC      480
GGCTGGTGTG CACGGACCCCT GGATGCCCCG ACGCGTACCA CAACCCCCCC GACATGAAGA      540
CCCATGCCTG TGGCGGCAAC AGCAACTACC AAGTCACCTT CTGCCCGTGA TCGGCCGGAA      600
TAGGCTCTGC ATGCATGGGC TAGTGATTG GATTTTGCAG GAATAATTCT GATTTGGATT      660
TGCACAATAA TATATAAGCG TGCATTACAC GTGCAOGCAT TTGTATGTAC GCGCGTATAC      720
GTCGTACCTA CGTAGCTGGA TTTGTGTGCG TGCTTCGTAC CAAATATTAA TAAGAATAAA      780
CAAATATGAC TCGTGTGATA TATATATAAA AAAAAAAAAA AAAAACTCGA G      831

```

## (2) INFORMATION FOR SEQ ID NO: 32:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 172 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```

Met Ala Ala Ala Ser Ser Val Leu Leu Leu Leu Ala Ala Ala Leu
 1             5             10             15
Ala Gly Met Ser Ala Asn Ala Ala Thr Phe Thr Ile Thr Asn Asn Cys
      20             25             30
Gly Phe Thr Val Trp Pro Ala Ala Thr Pro Val Gly Gly Gly Thr Gln
      35             40             45
Leu Asn Pro Gly Gly Thr Trp Thr Val Asn Val Pro Ala Gly Thr Ser
      50             55             60
Ser Gly Arg Val Trp Gly Arg Thr Gly Cys Ser Phe Asn Gly Asn Ser
      65             70             75             80
Gly Ser Cys Gln Thr Gly Asp Cys Gly Gly Ala Leu Ala Cys Thr Leu
      85             90             95
Ser Gly Gln Pro Pro Leu Thr Leu Ala Glu Phe Thr Ile Gly Gly Ser
      100            105            110
Gln Asp Phe Tyr Asp Ile Ser Val Ile Asp Gly Tyr Asn Leu Ala Met
      115            120            125
Ala Phe Ser Cys Ser Thr Gly Val Arg Leu Val Cys Thr Asp Pro Gly
      130            135            140
Cys Pro Asp Ala Tyr His Asn Pro Pro Asp Met Lys Thr His Ala Cys
      145            150            155            160

```

- 73 -

Gly Gly Asn Ser Asn Tyr Gln Val Thr Phe Cys Pro  
 165 170

## (2) INFORMATION FOR SEQ ID NO: 33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 790 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..790
- (D) OTHER INFORMATION: /note= "cDNA called PSI-1"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```

GGCACGAGGT CATATTCGTT GAAAACATCA TTCACTCTTC TCTGTGTC TCCTCTTCAC      60
CCGAAATTGT CTCTGACACC GNCCTGNTNC AGNCATGGCT AATGCTGCGT CAGGAATGGC      120
AGTCCATGAT GACTGCWWRY KARKAKYYCY KGGAMTGAAG GCGAAAAGGA CACAMCRYTT      180
CATTGTCTAC AAGATTGAGG AGAAGCAGAA GCAAGTGATT GTTGAGAAAG TTGGTGAACC      240
TATTCTAACT TACGAGGACT TTGCAGCAAG TCTTCCAGCT GACGAATGMC GATACGCCAT      300
YTATGATTTC GACTTTGTCA CTGCAGAGAA TTGCCAGAAG AGCAAGATTT TCTTCATTGC      360
ATGGTGTCCC GACGTAGCAA AGGTGAGAAG CAAGATGATC TATGCGAGCT CTAAGGACAG      420
GTTCAAGCGT GAACTTGATG GAATTCAAGT GGAGCTTCAA GCAACTGATC CAACTGAGAT      480
GGATCTTGAT GTTTTGAAAA GCCGCGTCAA CTAAAAGAAA CTCTCTTTGA ATAAGCTTCC      540
GATTATATTC GACTATTATG AAATGGTGTT TTCTATTCTC TTACAGTCTC TTGTGACTGT      600
TAGATACACT TGACACCCCT CATTCATCTT GTCTGGTCTG TTCTCTGTGT CTATGTTACC      660
TTACAGTGTC TCATCTATAT TCAAAATTAT CACAAGGTTT GTGCTGTGTT TGCTTCCTTT      720
GTTTCAACTC TACCAGGCT TAAGATTAAG ATTTGGGTAA GATGATTCTT TTAAAAAAA      780
AAAAAAAAAA

```

## (2) INFORMATION FOR SEQ ID NO: 34:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 508 base pairs
- (B) TYPE: nucleic acid

- 74 -

- (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: misc feature  
(B) LOCATION:1..508  
(D) OTHER INFORMATION:/note= "cDNA called PSI-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CCTCCAAGAA CCTAGTGGGA TCCCCCGGC CTGCAGGAAT TCGGCACCGA GTCCTTGCC	60
GAAATTCGGC ACGAGTGTG ATCTAAATCC TCACTAGGTG GTTCGGTTTA AGCTTAGCAA	120
CGCCAAAGAT CTAGACAATA ATTGCAAGTT ACGCGGTGA TCCTATCTTC CGTCCGAAG	180
CCGTCCTTGA TCCAGCCCAA GATGTTTTGT GGTCAGATGA CAAAGTAGAT CCACGGGTAT	240
ATATTATGGT AAAGTTGGCG GCCGAAGGAT TGAAGTCAA TAATGATATG TTTCTGGTG	300
GTTGCATCCC ATCTGAAGTG GTTATAGCCC CCAAAAACAA AAAATCTCTA CAATTTTGAA	360
AGGTGTTTCGT GGCAGGAAAT CTGCTAAGCA AGGAAGGATC TCCAAGAATC CAACTGGGAA	420
TAGAAGGATC CATCAATCAT TCTCTAATGA GGAGGAATCT GTTTAACTTT GTTATTGACT	480
AATAATCCTA TTATCGCCTA AAGCTGTT	508

(2) INFORMATION FOR SEQ ID NO: 35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 570 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: misc feature  
(B) LOCATION:1..570  
(D) OTHER INFORMATION:/note= "cDNA called PSI-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GGCAGGAGCT CGTGCCGCTC GTGCCGAATT CGGCACGAGG CAACATTTGC AACACCATCG	60
ACGGTGATAG GCCTCGGAGG ATCATCCATC ACCACCAAAC CCTTCTCTTC ATCCTTTTTA	120

- 75 -

AAACCAACAT TAAGCGCCAA GAACCCTTTG AGACTCGCCG GTGCATCGGG AGGAAGAGTC	180
ACTTGCTTTG AGAGGAACTG GTTGAGGAGA GATTTGAACG TGGTAGGATT TGGGCTGATC	240
GGATGGCTAG CTCCGTCGAG CATTCCAGCG ATAAATGGGA AGAGCCTGAC GGGTCTCTTC	300
TTCGATAGCA TCGGAACTGA GCTCGCTCAC TTCCCGACTC CTCCAGCTCT CACTTCACAG	360
TTCTGGTTGT GGTGGTTAC GTGGCACTTA GGCTCTTCC TCTGCCTCAC TTTCGGACAA	420
ATCGGATTCA AGGGCAGGAC TGAAGATTAC TTCTAAGGAT AACTATTCTT GTTTTCGTTT	480
GTACTATATG CTCTCTCTGG KTATGTGTAA TATTATCAAT CAAAACAAAG CTTTCTTTGC	540
TATTTGATGT TTCATTCTAT AAAAAAAAAA	570

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1418 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..1418
- (D) OTHER INFORMATION: /note= "cDNA called PSI-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GGCACGAGCA GAATCCTTAG CATCAGAGAA TTCTTCAATG ACAGACACTT ATAACCAGCA	60
GAGAGGTCTT GTCAACCAAC TAAAAGATGA CATGGAGAGG CTATATCAAC AGATCCAAGC	120
CCAAATGGGC GAACTTGAGT CTGTCAGGGT TGAGTATGCA AACGCGCAGC TAGAATGTAA	180
TGCTGCTGAT GAGCGTTCCC AAATACTGGC TTCTGAAGTC ATCAGTTTGG AAGATAAGGC	240
TCTCAGACTC AGGATCTAAT GAGTTAAAGC TGGAGAGGGA ACTGGAGAAG GCACAAAACA	300
GAAATTGTTA TCTTACAAGA AAAAATTGCA GGAGCTTAGA GAAAGGATCC GTCAAAGATT	360
TACAAATCTT ACTATTAAAG CTCTTCAGGA AAGAGAAAGG AAGGGTCCCT ACAAACTAT	420
GGGTGCAGGA AAGCTTCATC TGGTGGGGAA AAATCCACTG GATCTTTGAG TTAAAACTCA	480
ACTAGCAGAA AGAACGTGTC AACCTCTACA GAAGTCTTGA CAATCTCAGA TACTACGCCA	540
GAGAGCTCCA ACCAGGAAAC AGATTCTACT ACTCTGCTCG AAAGTGATTG ATCTWATACA	600

- 76 -

GCTATCATTCTGAAACTAGACAATTAACCTTTGAAGGCTTTTCATTGAGCGTCCCAGCT	660
GATCAGATGAGAGTGATTCA TAACATTAAT ACGCTGATTG CTGAGTTGGC AATTGAGAAG	720
GAAGAACTGG TGCAAGCACT GTCATCTGAG TTATCTCGAA GTGCGCATGT GCAGGAGCTG	780
AACAAAGAGT TATCCAGAAA ACTTGAAGCG CAGACGCAAA GGTTAGAGCT TGTAACAGCG	840
CAGAAGATGG CCATAGACAA TGTTTCACCA GAAAAGCAGC AGSCGGACAC TCATGTTGTT	900
CAAGAGAGAA CMCCGATTGC AGATGAAGGC GATGAGGTGG TAGAAAGGGT TCTWAGGATG	960
GATCATGAAG ATGTTCCCAG GAGGACCGTC GAAAAGAAGG ACAAGCAAGC TTCTCTAATG	1020
CATGGATGGC TTGCTCAGTC GTGACCCTTG TTGCCATTCT GTATATCCCG GGCACAGCCA	1080
TGGATTGCTT CTTCTCCTTT AGKKKTGGAA GAATTCATTT GGTTCCTTTG ATCCCCAAAT	1140
TTTGGATTTC ATTGGATTGA GCMCAAATGG TATAGTGAGA AACACCATGA ACTGAAGAG	1200
TTCCAAATGG ATAGAGGAGA CTCGGAAGCT TAAACAGAGA GGAAGGTAGT TGTATATGA	1260
GTGCGTTTTT GTCTGAATTT TGTAATTGTT GAATATATAG CCAAGTTAAA GTGTCCTCC	1320
TAGCAAATAT AATTTACCAA GAAAAATCT TGTATGGTTT CGGATATATT TTATCTTCTC	1380
GTTTTAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	1418

## (2) INFORMATION FOR SEQ ID NO: 37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 913 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..913
- (D) OTHER INFORMATION: /note= "cDNA called PSI-5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GGCACGAGAG ACAATCAGAT TCTTTACTCT TACAACGGTG GAGACCAAAC CAACGAGAGT	60
CTTGCTGCTC TGTGTTTAGA AAAGTCTCCT CCTTTTCATA CTGGTIACIT TGAACTATT	120
GGTAAAAGAA GATTTGGGTT TTTAATTGGA GATGGGTTTG TTTATTTGCG CATTGTTGAT	180
GAGGTTTGA AGAGATCTAG TGTTCTTAAG TTTCTTGAAC ATTTGGAGAG ATGAATTTAA	240

- 77 -

GAAAGCTGCT AGGGAGAATT CTAGAGGAAG TTTCCTGCT ATGATTGGTT CTATTAATGT	300
TGAAGATCAG CTTGTTCCCTG TTGTTACTAG ACTTATAGCT TCTCTTGAAC GTGTTGCTGA	360
GAGTAGTAGT AACAAATGAAT TGAAGAGTAG TAATCTTGGT GAACAAAGCG AAGGTTGAA	420
TTCTACTAAA GCTCCGTTGT TGGGGAGGTT AAGTAAGCAA GAGAAGAAGA AAGGGAAAGA	480
TCATGTGATT GAGCTTGAGG AACACAGGAA GTCTAATGAT AGAGGGAACA TAACCGATGA	540
TTCAGCGGGA GCTGGAACGT CGTTGGAGAA GGAGTGTGTG TCGAGTAGTG GACGTTCTGT	600
TACTCAAAGC TTGAATGGA AATGGCGGCG GYTAGTTCAG ATTGTTCTTG CTATTGATGC	660
AGCTATTTGC TTGACACTGT TTGGTATTTG GCTGGCTATA TGTCGGGGTA TCGAGTGAC	720
ACGTTGCTGA TTGTGCAGAC AGAAAATCGG ATAAACAGAG TCGATTCTTT ACATTCCCTT	780
GTGCATTCCA ATAGCCAAAA AATATGTTGA TTGTAATGTG AACCATTCTT AAGTTATAAT	840
TTGCCTTATG TATTGCTTTA GTCACAGCTT TTGAAATATG TACAGTCGGA TTCTAGAAAA	900
AAAAAAAAAA AAA	913

- 78 -

WHAT IS CLAIMED IS:

1. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13), wherein the wild-type gene corresponding to said SAR gene can be chemically induced in a plant in a protein-synthesis independent manner.
2. A DNA molecule according to claim 1, wherein the corresponding wild-type gene can be chemically induced in a plant despite the presence of the protein synthesis inhibitor cycloheximide.
3. A DNA molecule according to claim 1, wherein the corresponding wild-type gene can be induced in a plant by salicylic acid, isonicotinic acid or methyl benzo-1,2,3-thiadiazole-7-carboxylate.
4. A cDNA molecule according to claim 1.
5. A DNA molecule according to claim 1 comprising DNA selected from the group of DNA molecules p11.30.13 (SEQ ID NO. 11), p66B1 (SEQ ID NO. 14), pDPA2 (SEQ ID NO. 16), PSI-1 (SEQ ID NO. 34), PSI-2 (SEQ ID NO. 35), PSI-3 (SEQ ID NO. 36), PSI-4 (SEQ ID NO. 37), and PSI-5 (SEQ ID NO. 38).
6. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) obtainable by a method comprising
  - (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
  - (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.



- 79 -

7. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) obtainable by a method comprising
  - (a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
  - (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.
8. A recombinant or chimaeric DNA molecule comprising a plant SAR gene, wherein the wild-type gene corresponding to said SAR gene can be chemically induced in a plant in a protein-synthesis dependent manner.
9. A DNA molecule according to claim 8 wherein the corresponding wild-type gene can only be chemically induced in the absence of the protein synthesis inhibitor cycloheximide.
10. A DNA molecule according to claim 8 wherein the corresponding wild-type gene can be induced by salicylic acid, isonicotinic acid or methyl benzo-1,2,3-thiadiazole-7-carboxylate.
11. A cDNA molecule according to claims 8.
12. A DNA molecule according to claim 8 comprising DNA selected from the group of DNA molecules p1.1.1 (SEQ ID NO. 9), p.11.31.4 (SEQ ID NO. 10), and p.14.22.3 (SEQ ID NO. 15).
13. A recombinant or chimaeric DNA molecule comprising a plant SAR gene obtainable by a method comprising
  - (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;

- 80 -

- (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.
14. A recombinant or chimaeric DNA molecule comprising a plant SAR gene obtainable by a method comprising
- (a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
  - (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.
15. A recombinant or chimaeric DNA molecule comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a plant.
16. A DNA molecule according to claim 15 wherein the corresponding wild-type gene can be induced in a plant by methyl benzo-1,2,3-thiadiazole-7-carboxylate.
17. A DNA molecule according to claim 15 comprising DNA selected from the group of DNA molecules WCI-1 (SEQ ID NO. 3), WCI-2 (SEQ ID NO. 4 / SEQ ID NO. 5), WCI-3 (SEQ ID NO. 6), WCI-4 (SEQ ID NO. 7), and WCI-5 (SEQ ID NO. 8).
18. A recombinant or chimaeric DNA molecule encoding Arabidopsis class IV chitinase, Tobacco chitinase/lysozyme, Maize PR-1, or Maize PR-5.
19. A DNA molecule according to claim 18 encoding Arabidopsis class IV chitinase type A or B.

- 81 -

20. A DNA molecule according to claim 18 comprising the DNA sequence pChit4-TA (SEQ ID NO. 1), pChit4-TB (SEQ ID NO. 2), pBSCL2 (SEQ ID NO. 20), or pBSTCL226 (SEQ ID NO. 21).
21. A DNA molecule according to claim 18 comprising the DNA sequence PR-1mz (SEQ ID NO. 30), or PR-5mz (SEQ ID NO. 32).
22. A recombinant or chimaeric DNA molecule comprising the promoter region of the Arabidopsis PR-1 gene.
23. A DNA molecule according to claim 18 comprising 4.2 kb upstream of the start codon of the PR-1 wild-type gene.
24. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis independent manner, comprising
  - (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
  - (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.
25. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis independent manner, comprising
  - (a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
  - (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

- 82 -

- (c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.
26. A method according to claims 24 or 25, wherein cycloheximide is used as the protein synthesis inhibitor.
27. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis dependent manner, comprising
- (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
  - (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.
28. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis dependent manner, comprising
- (a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
  - (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
  - (c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.
29. A method according to claims 27 or 28, wherein cycloheximide is used as the protein synthesis inhibitor.
30. A method for obtaining a recombinant or chimaeric DNA molecule comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a wheat plant, comprising

- 83 -

- (a) cloning genes which are chemically inducible by differential screening of wheat cDNA libraries;
  - (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from wheat cells chemically induced; and
  - (c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.
31. A method for obtaining a recombinant or chimaeric DNA molecule encoding plant class IV chitinase comprising amplifying by the polymerase chain reaction DNA molecules from plant DNA using 5' and 3' oligonucleotides designed degenerate for the peptides HFCYIEE (SEQ ID NO. 27) and IRAING (SEQ ID NO. 28), respectively.
32. A method for obtaining a recombinant or chimaeric DNA molecule encoding plant chitinase/lysozyme comprising screening a plant cDNA library with a cDNA probe encoding cucumber chitinase/lysozyme.
33. A method for obtaining a recombinant or chimaeric DNA molecule comprising the promoter region of the Arabidopsis PR-1 gene comprising
- (a) screening of an Arabidopsis cDNA library with a probe of Arabidopsis PR-1 c-DNA; and
  - (b) identifying a clone carrying the promoter fragment by hybridisation to an oligonucleotide corresponding to coding sequences of the Arabidopsis PR-1 gene.
34. A method of improving protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins exert a synergistic effect.
35. A method according to claim 34, wherein two or more DNA molecules according to any one of claims 1-19 are transgenically expressed.
36. A method of protecting plants against pests comprising transgenically expressing in said plant class III chitinase.
37. A method according to claim 36, wherein tobacco plant chitinase III is transgenically expressed.

- 84 -

38. A transgenic plant and the progeny thereof comprising a DNA molecule according to any one of claims 1 to 23 displaying anti-pathogenic properties.
39. A transgenic plant according to claim 38 expressing transgenic Tobacco chitinase/lysozyme.
40. A transgenic plant and the progeny thereof comprising two or more DNA molecules encoding anti-pathogenic proteins, which plant displays synergistic anti-pathogenic properties.
41. A transgenic plant according to claim 40 comprising two or more DNA molecules according to any one of claims 1-23.
42. Seed of a transgenic plant according to any one of claims 38-41.
43. Use of a DNA molecule according to any one of claims 1 to 7 to achieve expression of a protein-synthesis independent plant SAR gene in transgenic plants.
44. Use of a DNA molecule according to any one of claims 8 to 14 to achieve expression of a protein-synthesis dependent plant SAR gene in transgenic plants.
45. Use of a recombinant or chimaeric DNA molecule according to any one of claims 18 to 21 to obtain transgenic plants displaying anti-pathogenic properties.
46. Use of the promoter region according to claim 22 to chemically induce a gene in operable linkage with said promoter.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: <b>C12N 15/82, 15/29, C07K 14/415, A01N 63/02, A01H 5/00</b>		(11) International Publication Number: <b>WO 95/19443</b>
<b>A3</b>		(43) International Publication Date: <b>20 July 1995 (20.07.95)</b>
(21) International Application Number: <b>PCT/IB95/00002</b> (22) International Filing Date: <b>3 January 1995 (03.01.95)</b> (30) Priority Data: <b>08/181,271</b> <b>13 January 1994 (13.01.94)</b> <b>US</b> (60) Parent Application or Grant (63) Related by Continuation <b>US</b> <b>08/181,271 (CIP)</b> <b>Filed on</b> <b>13 January 1994 (13.01.94)</b> (71) Applicant (for all designated States except US): <b>CIBA-GEIGY AG [CH/CH]; Klybeckstrasse 141, CH-4002 Basle (CH).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>RYALS, John, A. [US/US]; 14 Sanderling Court, Durham, NC 27713 (US). ALEXANDER, Danny, C. [US/US]; 217 Midenhall Way, Cary, NC 27513 (US). UKNES, Scott, J. [US/US]; 1003 Pinedale Drive, Apex, NC 27502 (US). WARD, Eric, R. [US/CH]; Säckerstrasse 20, CH-4058 Basle (CH).</b> (74) Common Representative: <b>CIBA-GEIGY AG; Patentabteilung, Klybeckstrasse 141, CH-4002 Basle (CH).</b>		(81) Designated States: <b>AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</b>  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  (88) Date of publication of the international search report: <b>12 October 1995 (12.10.95)</b>
(54) Title: <b>CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES AND USES THEREOF</b>		
(57) Abstract  <p>The present invention provides recombinant or chimaeric DNA molecules comprising plant SAR genes, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis independent or in a protein-synthesis dependent manner. Methods for obtaining these DNA molecules are also provided. Additionally, the present invention provides chemically inducible wheat genes, Arabidopsis chitinase IV, Maize PR-1mz, and Maize thaumatin PR-5mz. The anti-pathogenic sequences according to the invention can be genetically engineered and transformed into plants to obtain transgenic plants with enhanced resistance to disease. In one embodiment of the present invention, the chemically regulatable DNA promoter sequence of the Arabidopsis Pr-1 gene is provided. Another aspect of the present invention is a method of improving protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins exert a synergistic effect.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				



# INTERNATIONAL SEARCH REPORT

Inter. Application No.  
PCT/IB 95/00002

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/82 C12N15/29 C07K14/415 A01N63/02 A01H5/00		
According to International Patent Classification (IPC) or to both national classification and IPC:		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A01N A01H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATABASE RELEASE 36. ACCESSION NO. T04423. 30-8-93. NEWMAN, T. 470 ARABIDOPSIS THALIANA CDNA CLONE 40E6T7 see sequence ---	1-7
A	EMBL SEQUENCE DATABASE RELEASE 36. ACCESSION NO. Z26030. 8-9-93. QUIGLEY AND MACHE R. A. THALIANA TRANSCRIBED SEQUENCES. CLONE GBGE170 see sequence ---	1-7
X	EMBL SEQUENCE DATABASE RELEASE 36. ACCESSION NO. T04095. 30-8-93. NEWMAN, T., 45 ARABIDOPSIS THALIANA CDNA CLONE SCF12T7P see sequence --- -/--	1-7
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<div style="display: flex;"> <div style="width: 50%;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center;">7 June 1995</div>		Date of mailing of the international search report  <div style="text-align: center;">12. 09. 95</div>
Name and mailing address of the ISA European Patent Office, P.O. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. ( + 31-70 ) 340-2040, Tx. 31 651 epo nl, Fax: ( + 31-70 ) 340-3016		Authorized officer  <div style="text-align: center;">MADDOX, A</div>

## INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/IB 95/00002

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATABASE RELEASE 37. ACCESSION NO. Z26429. 24-9-93. PARMENTIER Y., ET AL., A. THALIANA TRANSCRIBED SEQUENCE; CLONE FAFC80-1. see sequence ---	1-7
X	EMBL SEQUENCE DATABASE RELEASE 36. ACC. NO. T04101; NEWMAN T.; 51 ARABIDOPSIS THALIANA CDNA CLONE SCG7T7P. 30-8-93. see sequence ---	1-7
A	THE PLANT CELL, vol.4, 2 pages 645 - 656 UKNES, S., ET AL. 'ACQUIRED RESISTANCE IN ARABIDOPSIS' see page 649 - page 651 ---	1-7
A	THE PLANT CELL, vol.3, 19 October 0 pages 1085 - 1094 WARD, E.R., ET AL. 'COORDINATE GENE ACTIVITY IN RESPONSE TO AGENTS THAT INDUCE SYSTEMIC ACQUIRED RESISTANCE' see page 1087 - page 1089 ---	1-7
A	DEVELOPMENTS IN PLANT PATHOLOGY. VOL. 2. MECHANISMS OF PLANT DEFENSE RESPONSES. 2ND INTERNATIONAL CONFERENCE OF THE EUROPEAN FOUNDATION FOR PLANT PATHOLOGY, STRASBOURG, FRANCE, AUGUST 24-27, 1992. KLUWER ACADEMIC PUBLISHERS, pages 422 - 432 LAWTON, K., ET AL. 'THE MOLECULAR BIOLOGY OF SYSTEMIC ACQUIRED RESISTANCE' see page 424 - page 426 ---	34, 35, 38, 40-43
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, 1 August 1993, WASHINGTON US pages 7327 - 7331 ALEXANDER, D., ET AL. 'INCREASED TOLERANCE TO TWO OOMYCETE PATHOGENS IN TRANSGENIC TOBACCO EXPRESSING PATHOGENESIS RELATED-PROTEIN 1A' see page 7329 - page 7331 ---	34, 35, 38, 40-43
A	THE PLANT CELL, vol.5, 3 pages 159 - 169 UKNES, S., ET AL. 'REGULATION OF PATHOGENESIS-RELATED PROTEIN-1A GENE EXPRESSION IN TOBACCO' see abstract --- -/--	34, 35, 38, 40-43

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IB 95/00002

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 392 225 (CIBA GEIGY AG) 17 October 1990  see the whole document ---	1-7, 24-26, 34, 35, 38, 40-43
A	EP,A,0 332 104 (CIBA GEIGY AG) 13 September 1989  see the whole document -----	1-7, 24-26, 34, 35, 38, 40-43

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 95/00002

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 34, 35, 38, 40-42  
because they relate to subject matter not required to be searched by this Authority, namely:  
- see remark on additional sheet
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- see remark on (4) additional sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7, 24-26, 43 (all completely); 34, 35, 38, 40-42 (all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

The single problem underlying the the proposed solutions of the present application , is the provision of chemically regulatable SAR genes and their use in transgenic plants .

The solution proposed in its broadest form is the provision of a number inducible SAR gene from various sources (SEQ. I.D.'S 1-11,14-16,20,21,29,31,33-37 as defined at page 13 of the description), and the use of SAR genes in crop protection and gene regulation. However chemically inducible SAR genes are known from the prior art, see for example: The Plant Cell vol. 4, pp.645-656 (1992) (=D1), see pages 649-651; The Plant Cell vol.3, pp.1085-1094 (1991) (=D2), see pages 1087-1089. Furthermore their use in transgenic plants is also known from the prior art, see for example: Developments in Plant Pathology vol. 2 (1993), eds Fritig, B., et al., pp.422-432 (=D3) see pages 424-426; Proc. Natl. Acad. Sci. (USA), vol. 90, pp.7327-7331 (1993) (=D4), see pages 7329-7380; The Plant Cell, vol. 5, pp.159-169 (1993) (=D5), see abstract page 159.

In the light of the prior art documents, D1 to D5 inclusive, the problem underlying the present invention can be redefined as providing further inducible SAR genes and, independantly providing further means for the effective use of SAR genes. The solutions to these redefined problem are the sequences represented by SEQ. I.D.'S 1-11,14-16,20,21,29,31,33-37, the use of synergistic SAR compositions, and the Arabidopsis PR-1 promoter and its use in gene regulation.

The ISA considers that due to the fact that there are existing solutions to the problem as shown above and due to the essentially different nature of the solutions provided by the application, and furthermore that no other technical feature can be distinguished common to all these solutions, which can be regarded as special, the ISA is of the opinion that there is no single inventive concept underlying the plurality of claimed inventions of the present application within the sense of Rule 13.1 PCT.

However taking into account the balance between necessary search effort and the levying of additional fees, the ISA has been able to regroup the different claimed inventions (on the basis of certain independant technical features) into eight inventions, formulated as different subjects in the communication pursuant to Article 17(3)(a) PCT.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. Claims 1-7, 24-26, and 43 all completely, (34, 35, 38, 40, 41, and 42 all partially), : Protein synthesis independant chemically inducible SAR genes as represented by the SEQ. I.D.'S 11, 14, 16, 33-37, methods for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far as they relate to these sequences or combinations thereof. (note that SEQ I.D.'S are derived from the description at page 13, since the I.D. NOS. as defined by claim 5 would appear to be incorrect)

2: Claims 8-14, 27-29, and 44 all completely, (34, 35, 38, 40, 41, and 42 all partially), : Protein synthesis dependant chemically inducible SAR genes as represented by the SEQ. I.D.'S 9, 10, 15, methods for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far they relate to these sequences or combinations thereof.

3. Claims 15-17, and 30 all completely, (34, 35, 38, 40, 41, and 42 all partially): Chemically inducible wheat genes, as represented by SEQ. I.D.'S 3-3, method for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far as they relate to sequences or combinations thereof.

4. Claims 19, 31, both completely, (18, 20, 34, 35, 38, 40, 41, 42, and 45 all partially): Arabidopsis chitinase IV genes as represented by SEQ. I.D.'S 1 and 2, method for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequences or combinations thereof.

5. Claims 32, 36, 37, and 39 all completely, (18, 20, 34, 35, 38, 40, 41, 42, 45 all partially): Chitinase III, chitinase/lysozyme genes as represented by SEQ. I.D.'S 20 and 21, method for their isolation and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequences or combinations thereof.

6. Claims 21 completely, (18, 34, 35, 38, 40, 41, 42, 45 all partially): Maize PR-1 and PR-5 genes as represented by SEQ. I.D.'S 29 and 31, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequences or combinations thereof. (note: the SEQ. I.D.'S are derived from the description at page 13, since the I.D. NOS. as defined by claim 21 refer to amino acid sequences not DNA sequences)

7. Claims 22, 23, 33, 46, all completely, and (38 and 42 both partially): Arabidopsis PR-1 promoter, method for its isolation, and its use for chemical induction of gene expression, and antipathogenic use in transgenic plants. Claims 38 and 42 partially in so far as they relate to this promoter.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

8. Claims 34, 40 and 42 all partially: Method for improving protection against pests in transgenic plants by the expression of two or more gene products with synergistic effect, transgenic plants and seeds. Partially in so far as they relate to combinations not involving any of the sequences of the above mentioned groups.

The search has been carried out as follows

Yes

1. Claims 1-7, 24-26, and 43 all completely, (34, 35, 38, 40, 41, and 42 all partially): Protein synthesis independent chemically inducible SAR genes as represented by the SEQ. I.D.'S 11, 14, 16, 33-37, methods for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far as they relate to these sequence I.D.'s or combinations thereof. (note that SEQ I.D.'S are derived from the description at page 13, since the I.D. NOS. as defined by claim 5 would appear to be incorrect)

No

2. Claims 8-14, 27-29, and 44 all completely, (34, 35, 38, 40, 41, and 42 all partially): Protein synthesis dependant chemically inducible SAR genes as represented by the SEQ. I.D.'S 9, 10, 15, methods for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far as they relate to these sequence I.D.'s or combinations thereof.

No

3. Claims 15-17, and 30 all completely, (34, 35, 38, 40, 41, and 42 all partially): Chemically inducible wheat genes, as represented by SEQ. I.D.'S 3-3, method for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41 and 42 partially in so far as they relate to sequence I.D.'s or combinations thereof.

No

4. Claims 19, 31, both completely, (18, 20, 34, 35, 38, 40, 41, 42, and 45 all partially):

Arabidopsis chitinase 17 genes as represented by SEQ. I.D.'S 1 and 2, method for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequence I.D.'s or combinations thereof.

No

5. Claims 32, 36, 37, and 39 all completely, (18, 20, 34, 35, 38, 40, 41, 42, 45 all partially): Chitinase, chitinase/lysozyme genes as represented by SEQ. I.D.'S 20 and 21, method for their isolation, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequence I.D.'s or combinations thereof.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

No  
5. Claims 21 completely, (18, 34, 35, 38, 40, 41, 42, 45 all partially): Maize PR-1 and PR-5 genes as represented by SEQ. I.D.'S 29 and 31, and their antipathogenic use in transgenic plants. Claims 34, 35, 38, 40, 41, 42 and 45 partially in so far as they relate to these sequence I.D.'s or combinations thereof. (note the SEQ. I.D.'S are derived from the description at page 13, since the I.D. NOS. as defined by claim 21 refer to amino acid sequences not DNA sequences)

No  
7. Claims 22, 23, 33, 46, all completely, and (38 and 42 both partially): Arabidopsis PR-1 promoter, method for its isolation, and its use for chemical induction of gene expression, and antipathogenic use in transgenic plants. Claims 38 and 42 partially in so far as they relate to this promoter.

No  
3. Claims 34, 40 and 42 all partially: Method for improving protection against pests in transgenic plants by the expression of two or more gene products with synergistic effect, transgenic plants and seeds. Partially in so far as they relate to combinations not involving any of the sequences of the above mentioned groups.

REMARK: Since there is no evidence for the antipathogenic activity, or the synergistic actions of the gene products covered by the first invention as formulated in the communication pursuant to Article 17(3)(a) PCT, the claims 34, 35, 38, 40, 41, 42 have only been searched to the extent that such features and transgenic plants displaying such properties would have been retrieved on searching for the genes themselves.



# INTERNATIONAL SEARCH REPORT

information on patent family members

Inter      nal Application No  
PCT/IB 95/00002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0392225	17-10-90	AU-B- 642865	04-11-93
		AU-A- 5218390	27-09-90
		CA-A- 2012778	24-09-90
		JP-A- 3035783	15-02-91
-----			
EP-A-0332104	13-09-89	AU-A- 3108089	14-09-89
		JP-A- 2009377	12-01-90
		PL-B- 162317	30-09-93
		PT-B- 89915	31-10-94
-----			

